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13. ABSTRACT (Maximum 200 Words) The major objective of this proposal is to investigate the consequences of long-term, low-level exposure to sarin, alone, and in combination with pyridostigmine bromide (PB) and stress on the development of chronic and neurologic deficits. An important finding from our studies is that brain AChE and plasma BChE inhibition might be used as a biomarker for exposure, not for sarin-induced neurologic deficits. Rats treated with toxic doses of sarin showed inhibition of brain and plasma cholinesterases, that subsequently recovered. On the other hand, these animals exhibited sensorimotor deficit that persisted throughout the six-month observation period. Another significant result from our studies is that a brief insult from a single lethal or sub-lethal dose of sarin, can generate a series of events to result in a diffuse neuronal cell death. Neuronal degeneration was exhibited in the cerebral cortex, the hippocampus, and Purkinje cell neurons in the cerebellum. Stress, caused by immobilization or heat also exacerbated sarin-induced neurotoxicity. The results show that prophylactic treatment with PB offered some protection of peripheral AChE against sarin inhibition. On the other hand, PB exacerbated the toxicity of high doses of sarin. Furthermore, the results showed that treatment with either sarin or PB alone resulted in sensorimotor impairments that persisted throughout the six-month observation period. Neuronal cell death of the motor cortex and Purkinje cells of the cerebellum following exposure to sarin could lead to weakness and loss of strength as well as problems with gait and coordination of movement. Further, disruption of hippocampal circuitry due to degeneration of neurons in different subfields can lead to learning and memory deficits. The above changes likely explain some of the symptoms such as loss of memory, muscle weakness, and alterations in learning ability observed in Gulf War Veterans.				
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FOREWORD

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10/23/02

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Introduction

The Nature of the Problem

The goal of this project is to investigate the long-term neurological dysfunction, the neurochemical and neuropathological consequences following low-level, sub-clinical exposure to nerve agent sarin, alone and in combination with pyridostigmine bromide (PB) and environmental factors such as stress and heat. The hypothesis is that exposure to low-dose sarin in combination with prophylactic drug, pyridostigmine bromide (PB), and stress would lead to exacerbated neurotoxic effects than that caused by sarin alone. The specific aims of the study are as follows;

Specific Aim 1. Dose range-finding study.

Specific Aim 2. Effect of stress on sub-clinical sarin exposure.

Specific Aim 3. Effect of heat on sub-clinical sarin exposure.

Specific Aim 4. Effect of prophylactic treatment with pyridostigmine bromide (PB) on sub-clinical sarin exposure.

Specific Aim 5. Effect of treatment with PB after sub-clinical sarin exposure.

Specific Aim 6. Effect of stress and prophylactic treatment with PB on sub-clinical sarin exposure.

Background

Test Compounds/Environmental Factors:

Sarin:

Sarin, *O*-isopropylmethylphosphonofluoridate, is an organophosphate that has been studied primarily as a potent nerve agent for warfare purposes (Taylor, 1985). The main clinical

features associated with acute sarin intoxication are seizures, fasciculations, tremors and hypothermia (Taylor, 1985). The appearance of these symptoms correlates with the inhibition of AChE, both in the CNS and peripheral nervous system (PNS) (Gupta et. al., 1991). This is followed by excessive accumulation of acetylcholine at the nicotinic and muscarinic acetylcholine receptors, leading to hyperactivation of the receptors. Excessive accumulation of acetylcholine leads to activation of ligand-gated ion channel, nicotinic acetylcholine receptor (nAChR) and muscarinic acetylcholine receptor (mAChR). These receptors mediate diverse cellular responses by distinct signaling mechanisms (Wess, 1996). Indeed previous studies from our laboratory and others have shown that organophosphate compounds including sarin cause differential regulation of nAChR and mAChR (Huff et. al., 1994, Katz et. al., 1997, Khan et al, 2000). *In vitro* studies by Bakry et al (1988) suggested that sarin binds to nAChR and modulates its ligand binding characteristics. Therefore, it is likely that changes in cholinergic pathways would play a key role in the toxicity induced by sarin.

In addition to acute cholinergic effects, healthy individuals exposed to low dose sarin have been reported to exhibit neurological signs and symptoms up to 10 years following initial exposure (Sidell, 1974; Duffy and Burchfiel, 1980). Abnormal electrophysiological recordings following a single large doses or repeated subclinical dose of sarin in rhesus monkeys have been observed (Burchfiel et al., 1976; Burchfiel and Duffy, 1982).

Pyridostigmine Bromide (PB):

PB is a quaternary dimethyl carbamate that has been used for the treatment of myasthenia gravis (Breyer-Pfaff et al., 1985). It was given to PGW veterans for prophylactic protection to shield acetylcholinesterase (AChE) from the nerve agent poisoning. PB acts by reversibly

inhibiting 30-40% of the AChE in the peripheral nervous system, thus protecting the enzyme from irreversible inhibition by nerve agents (Blick *et al.*, 1991). The enzyme activity is restored following spontaneous decarbamylation resulting in near normal neuromuscular and autonomic functions (Blick, *et al.*, 1991). Toxic symptoms associated with PB overdose are primarily associated with over-stimulation of nicotinic and muscarinic receptors in the peripheral nervous system resulting in exaggerated cholinergic effects such as muscle fasciculations, cramps, weakness, muscle twitching, tremors, respiratory difficulty, gastro-intestinal tract disturbances and paralysis (Abou-Donia, 1994). The major metabolic product of PB is 3-hydroxy-N-methylpyridinium resulting from the carbamate hydrolysis that abolishes its cholinergic action (Kornfeld *et al.*, 1970, 1971). Central nervous system effects of PB are not observed unless blood-brain barrier (BBB) permeability is compromised, because PB does not cross the BBB owing to the positive charge on the quaternary pyridinyl nitrogen atom (Birtley, *et al.*, 1966). Recent studies from different group of laboratories suggest that PB does not cross the BBB under stress (Lallement *et al.*, 1998; Sinton *et al.*, 2000; Grauer *et al.*, 2000; Kant *et al.*, 2001; Tian *et al.*, 2002; Song *et al.*, 2002). These studies are, however at variance with the studies by Friedman *et al.* (1996) that suggested an increase in the BBB permeability causing PB-induced effects in the CNS.

Stress:

While serving in the Persian Gulf War, the military personnel were exposed to a variety of environmental conditions that may have played an important role in the development of unique symptoms associated with their deployment in the region (Institute of Medicine, 1995, Nisenbaum *et al.*, 2000). Sustained stress is known to cause a variety of pathologic effects that may have detrimental bearing on the health and well being of an individual (Sapolsky, 1996).

Hippocampal damage and therefore, a deterioration in learning and memory has been observed following increase in cortisol levels (McEwen, 1998). The increased cortisol levels usually correlates well with the onset of stress. It has been reported that a few days of continuous stress causes a significant impairment in the ability of hippocampal neurons to survive seizures and ischemia, and following prolonged stress prominent neuronal loss occurs in hippocampus (Herman and Cullinan, 1997). Furthermore, Baker et al (1997) observed a strong correlation between posttraumatic stress disorder and self-reported physical symptoms in PGW veterans. Therefore, it is important to evaluate the long-term neurotoxic effects of sarin, alone, and in combination with other chemicals and stress.

Cholinergic system in the CNS plays an important role in learning and memory and has a regulatory role in certain neurobehavioral functions (Levey et al, 1995, Nostrandt et al, 1997). Excessive accumulation of acetylcholine following inhibition of AChE in the PNS or CNS following exposure with chemicals, such as organophosphorus compounds, including sarin, that primarily affect the cholinergic system, causes increased stimulation of the receptors targeted by acetylcholine. Under acute exposure conditions, over-stimulation leads to seizure and chronic activation leads to impairment of memory and neurobehavioral performance (Taylor, 1985).

CNS cyto-architecture is maintained by a complex cellular milieu that involves neurons and a variety of cells of astrocytic and glial origin. In order for the CNS to function properly and to respond to external stimuli, it is absolutely required that a proper communication is maintained within these cells. A major determinant of neuronal morphology is the cytoskeleton. Different components of cytoskeleton within the neurons and astrocytes provide forces to maintain the appropriate cellular structure, e.g. neuronal dendrites and axons are maintained in

stable conditions by the force provided by the elements of cytoskeleton (Gavin, 1997). Such interactions are essential for proper synapse formation. The components of cytoskeleton are microfilament, intermediate filaments and microtubules. An important neuronal component, MAP-2 is enriched in dendrites and cell bodies (Tucker et al., 1988), in which it stabilizes the polymerized tubulin. Abnormal regulation of expression of MAP-2 causes suppression of neurite outgrowth and reduction in number of neurites and dendrites in cultured neurons (Caceres et al., 1992). Similarly, aberrant intermediate filament proteins have been linked to diseases of neurodegeneration (Eliasson et al., 1999). GFAP and vimentin constitute the major component of astrocytic intermediate filament (Penky et al, 1999). GFAP is upregulated in response to reactive gliosis as a consequence of a variety of insults, such as exposure to neurotoxic chemicals, trauma, and neurodegenerative diseases that affect the CNS (Eng and Ghirmikar, 1994). The functions of GFAP and vimentin are not well understood, but it has been suggested to play an important role in long-term maintenance of brain cyto-architecture (Liedtke et al., 1996, Little et al, 1998), proper function of BBB (Pekny et al., 1998), and modulation of neuronal functions (Shibuki et al, 1996)

Microglia are involved in brain function under both normal and pathological conditions (Dickson et al, 1993, McGeer et al, 1993). In normal brain, resident ramified/resting microglia are activated to become rod-shaped or amoeboid shape in response to injury or toxic insult. Activated microglia proliferate, engulfing degenerating elements (Giulian et al, 1989, Stoll et al, 1989), while secreting cytotoxic agents that induce neuronal death and demyelination (Thery et al, 1991, Giulian et al, 1994).

Oxidative stress resulting from environmental chemicals has been considered as a cause of chemical exposure-related diseases. Apoptosis induced by environmental chemicals and neurotoxins plays a major role in oxidative stress resulting following treatment with toxic agents (Majno and Joris, 1995; Wyllie et al, 1984; Thompson, 1995). In the CNS, oxidative DNA damage has been observed following ischemia (Cui et al., 2000). Oxidative stress is known to cause an increased generation of single stranded DNA (ssDNA), which subsequently leads to increased DNA fragmentation and cell death by apoptosis and necrosis (Chen et al., 1997). An increase in the generation of 8-hydroxy-2'-deoxyguanosine and 3-nitrotyrosine has been observed due to chemical exposure (Kasai, 1997; Hensely et al, 1998; Maruyama et al, 1996). In response to toxic insult, when there is excessive DNA damage and repair is incomplete, cellular signaling pathways may be activated that may result in apoptosis (Halliwell and Arouma, 1991; Lowe et al, 1993). Thus, it is apparent that a diverse mechanism could be activated in response to toxic insult that may result in pathological damages and changes in the formation of ssDNA reflecting increased generation of oxidative stress and apoptosis.

Body:

A) Approach

Specific Aim 1. Dose range-finding study.

Study design: Male Sprague-Dawley rats (225-250g) were obtained from Zivic-Miller Laboratories, Allison Park, PA. The animals were housed in Duke University Medical Center vivarium at 21-23 °C and on a 12-h dark-light cycle. The animals were allowed food and water *ad libitum*. All the treatments and procedures on the animals were carried out strictly according

to the recommended guidelines by the Duke University Institutional Animal Care and Use Committee and U.S. Department of Defense.

Dose-response studies ranging following single i.m. treatment with sarin ranging from 0.01-1x LD50 under acute and sub-chronic (3 months) and chronic (6 months) conditions were carried out. Following acute exposure with a single i.m. injection of 0.01-1x LD50 sarin, there was differential regulation of plasma and central nervous system cholinesterase activity, changes in cortex and brainstem receptor ligand binding for nicotinic and muscarinic acetylcholine receptors (Khan et al, 2000). Sub-chronic studies were carried out after 90 days of single i.m. injection with 0.01-1x LD50 sarin. There was a significant decrease (~58% of control) in the blood-brain barrier (BBB) permeability in the brainstem, whereas no significant changes were observed in cortex, midbrain and cerebellum. There was an increase in the blood-testis barrier (BTB) permeability. Acetylcholinesterase activity in the cortex showed a decrease (~30% of control), whereas there was an increase (~20% of control) in the brainstem at 1xLD50 sarin (Jones et al, 2000).

For the studies on the chronic effects after single exposure with sarin, Male Sprague-Dawley (250-300 g) rats were exposed to single i.m injection of 1, 10, 50 or 100µg/kg (0.01-1xLD50) sarin. Control animals received equivalent volume of normal saline. Their sensorimotor functions were evaluated at 3 months and 6 months using a battery of tests that included measurements of postural reflexes, limb placing, grip time, beam walking and incline plane performance. The data from behavioral observations show that there was a significant deficit in incline plane performance, grip time, beam-walk score and beam-walk time at 3 months that progressively deteriorated further by 6 months at each dose of sarin.

For the neuropathological studies following acute exposure with sarin, two groups of male Sprague-Dawley rats were exposed to single i.m. dose of sarin (0.01, 0.1, 0.5, and 1x LD50 equivalent to 1, 10, 50, and 100µg/kg). Twenty four hrs after sarin treatment, one group of rats was sacrificed for biochemical determinations of cholinergic system and blood-brain barrier (BBB) evaluation and a second group was perfused under anesthesia for pathological evaluation by H&E staining and immunohistochemical analysis for BBB permeability, neuronal and glial integrity. Sarin treatment resulted in significant impairment in the BBB permeability only at 0.5 and 1xLD 50 doses. However, there was no BBB permeability or any pathological changes apparent at lower doses. MAP-2 immunostaining suggested neuronal dendritic loss in several brain regions (Abdel-Rahman et al, 2000).

In our efforts to study genetic basis of long-term neurological effects following subclinical low-dose exposure to sarin, we evaluated the expression profile of mRNA for GFAP and vimentin, α tubulin and acetylcholinesterase in various brain regions of rat. Following treatment with single i.m. dose of sarin (0.5xLD50, 50g/kg) at 1, 3 hrs, and 1, 3, and 7 days, there was a significant induction of mRNA expression for GFAP and vimentin in cortex, brainstem, midbrain, cerebellum and spinal cord. The expression of these genes showed differential pattern of expression in different brain regions. Both GFAP and vimentin mRNA were induced at early time point in all the regions except brainstem, where moderate to high levels were observed at 1 and 3 days, respectively following treatment with sarin. Expression of GFAP and vimentin remained at high levels in brainstem, cerebellum and midbrain at all the time periods, whereas expression pattern in the cortex and spinal cord returned to normal levels by 7 days post treatment. Similarly, α tubulin mRNA expression showed a differential pattern of early and late expression pattern in different brain regions (Damodaran et al, 2002; Damodaran et al, 2000;

Damodaran et al, in press). These data indicate that sarin-induced long-term abnormalities may involve changes in the gene expression pattern of the components of astroglial cytoskeleton and increased expression of acetylcholinesterase.

Electrophysiology Experiments

Rationale:

Exposures to neurotoxins can produce neuronal damage that may be reflected in electrophysiological changes. These changes can be detected *in vivo* or *in vitro*, depending on the nature of the neurotoxin, as well as the detection methods used. For example, *in vivo* tests have long been used to assess the damage produced by neurotoxin-induced seizures, for animals exposed to certain neurotoxins (e.g., pilocarpine) can go on to develop a chronic epileptic condition marked by recurrent spontaneous seizures detected with behavioral or electrophysiological tests (see review: Turski, et al., 1989). Furthermore, when brain slices are taken from such animals, *in vitro* electrophysiological recording may reveal evidence of brain damage, which may be manifested as alterations in normal neuronal function (e.g., loss of neuronal plasticity) or neuronal hyperexcitability ((e.g., epileptiform interictal spikes or seizure-like activity) (Nagao, et al., 1996)).

The neurotoxicity of sarin exposure was assessed using electrophysiological recording in hippocampal slices from 24 pairs of rats that were injected with sarin (treated) or saline (control). Two neurophysiological measures of toxicity were used: (1) long-term potentiation (LTP), a widely studied measure of hippocampal neuroplasticity and (2) the presence of excessive excitability in area CA3 of hippocampal slices when exposed to elevated extracellular potassium.

These two tests were chosen because they reflect different directions in which neural tissue can express toxicological damage: (1) the loss of normal cellular function (LTP) or (2) the

gain of abnormal cellular function (high potassium-induced hyperexcitability). Note that the underlying mechanisms for the latter (gain of function) actually could be a result of the loss of normal function (such as, for example, hyperexcitability resulting from a loss of normal GABAergic inhibition), nevertheless, the behavioral, functional expression will still be manifested as an apparent gain of abnormal activity.

Long-term potentiation (LTP):

Plasticity is an important property of normal neuronal tissue; it underlies brain development early in life, as well normal functions in adulthood, such as recovery from injury, as well as learning and memory. If a toxic insult disrupts the neuroplastic processes underlying learning and memory, severe behavioral disruption and disability may result. Accordingly, it is important to identify the underlying mechanisms of normal neuroplasticity so that neurotoxic targets can be identified. One such mechanism is long-term potentiation (LTP). LTP is an experimental phenomenon that has been proposed to relate to learning and memory (Bliss and Collingridge, 1993). In general, LTP is an activity-induced enhancement in a neuronal circuit. LTP has (1) been observed both *in vivo* and *in vitro*, (2) it occurs in a number of anatomical areas important for learning and memory and, (3) is disrupted by drugs that disrupt learning. Although there is still debate about the extent to which LTP relates to human learning and memory process, at the very least, LTP can be said to be a type of neuroplasticity that occurs in areas of the brain important for learning and memory. Thus, a toxic insult that disrupts LTP may disrupt learning and memory.

Previous studies have found changes in long term potentiation in toxin-exposed animals. Most relevant to our aims is the study by Armstrong, et al., (1997) in which rats were exposed to soman (injected once a day for 3-5 days), allowed to recover for 2 weeks, then LTP was

measured *in vivo*. There were three groups: one of control animals and two groups of soman-exposed animals. Of the treated rats, one group had been given doses of soman sufficient to produce seizures. The other group had been given soman injections that were stopped as soon as the animal exhibited tremors, but not seizures. (The rats with the higher dose behavioral changes chronically, manifest as “extreme hyperreactivity” noticed during handling 2 weeks after the exposure.) In both groups, LTP was impaired, but the most impaired rats were those in the group given enough soman to produce seizures. In addition, the authors report that the variability between soman-exposed animals was far more than in controls. This, they suggest, may have been due to the presence of undetected seizures in some of the treated groups. Thus, the presence of toxin-induced seizures may be a significant factor in the final outcome, and will be important in assessing animals in future studies.

Thus, if a neurotoxic insult due to an agent such as sarin (alone or in combination with other chemicals) appears to disrupt LTP, it is possible that this disruption predicts a more global disruption in behavior, such as in learning and memory.

High potassium-evoked hyperexcitability:

There are several *in vitro* electrophysiological methods in which to test for abnormal gain of function in toxin-exposed animals. One of which is to take brain slices from the exposed and control animals and then record the neuronal activity *in vitro*. If the animal has been exposed to a neurotoxin, there may be signs of damage expressed as hyperexcitability either in control artificial cerebrospinal fluid (ACSF) or in the presence of a near-threshold dose of a convulsant drug or conditions.

One such *in vitro* model is the elevated potassium ACSF model (“High K⁺-ACSF”) (Traynelis & Dingledine, 1988). In this model, the potassium concentration in the ACSF is

elevated from the baseline concentration (3.3-3.5 mM) to an elevated concentration (8.5 mM); this results in spontaneous bursting activity in hippocampal areas CA3 and CA1 and, in some slices, electrographic seizures. We have found that smaller elevations of potassium (e.g., to 5.0-7.0 mM) are near the threshold for this type of activity, for these levels do not always cause spontaneous bursting activity, especially in healthy, young adult tissue. However, if the neural tissue has been damaged, it may express abnormal activity even in near-threshold or slightly more elevated potassium concentrations in the ACSF. Accordingly, we used the High K^+ -ACSF model to assess the level of hyperexcitability in hippocampal slices from sarin- and saline-exposed rats. The animals were exposed to one dose of either LD_{50} or one-half- LD_{50} sarin (or saline) and allowed to recover for at least 4 weeks. This recovery time frame was chosen because it was close to the time frame used in the soman study (Armstrong *et al.*, (1997). In addition, we chose this time frame because, in some of the studies, we used the pairs of rats for two types of experiments: LTP and High K^+ -ACSF. In this way we were able to reduce the number of animals used for each type of electrophysiology study. Accordingly, we also wanted a weeks-long recovery time, because previous studies have reported that several weeks may be necessary for post-toxin-exposure hyperexcitability to be evident in electrophysiology studies. For example, Anderson, et al, (1999) found that a tetanus toxin injection to young (P10) rats caused acute behavioral seizures and then, if the animal is allowed to recover, *in vitro* electrophysiological signs of hyperexcitability in hippocampal slices 30 days after toxin exposure. They suggest that this delayed expression of hyperexcitability could be due to anatomical or functional remodeling processes that require several weeks to occur or be expressed (although the specific causal mechanism is still under study). It is possible that a similar process will occur in sarin-exposed adult rats.

Methods for long-term potentiation (LTP)

Rats were injected with either sarin or saline and allowed to recover for at least 4 weeks (range: 28-41 days post-injection). After the recovery period, each rat was weighed, anesthetized with halothane, decapitated, the brain was removed and then briefly chilled in iced ACSF. Using a vibratome, coronal slices (500 μ M) were cut in chilled ACSF. Slices were transferred to a holding chamber filled with 350 mls of oxygenated artificial cerebral spinal fluid (ACSF). The ACSF was composed as follows: 120mM NaCl; 3.3 mM KCl, 1.23 mM NaPO₄, 25 mM NaHCO₃, 0.9mM Mg SO₄, 2.0 mM CaCl₂, 10mM dextrose. Slices were allowed to recover at room temperature the holding chamber for at least 1 hour. Slices were then transferred to the brain slice recording chamber and allowed to equilibrate for 10 minutes at 29°C.

Long-term potentiation experiments were done as previously described (Barr, et al., 1995). Briefly, a glass extracellular recording electrode (filled with 150 mM NaCl, with a resistance of 1-2 megohms) was positioned in *stratum radiatum* of hippocampal area CA1 and a stimulating electrode (tungsten monopolar; AM Systems) was positioned nearby in *stratum radiatum* of area CA1. Oxygenated ACSF was superfused at approximately 2 mls/ minute onto the slice. A single stimulus was delivered every 30s; typically, excitatory post-synaptic potential (EPSP) evoked fields were obtained with a stimulus intensity from 40 μ A to 70 μ A. An input/output curve was performed at 1X intensity, 2X intensity, and 3X intensity (i.e., approximately 40 μ A, 80 μ A, and 120 μ A). A 10X intensity was used to determine the maximal baseline field slope, from which a baseline stimulus intensity was determined (i.e., 20 to 30 % of the maximal response was set as the baseline response).

LTP was induced as follows: slices were given a minimum of 20 minutes baseline stimuli to fully stabilize, then 3 theta-burst stimuli (TBS) were given (each 1 minute apart). Each

theta-burst consisted of 10 mini-trains of 4 pulses at 100 HZ, 200 ms apart. To determine the degree of potentiation (LTP), responses were measured for 20 to 30 minutes after last TBS. Responses were normalized with respect to the baseline EPSP slope; results were calculated from the last 10 minutes of the post-TBS period.

LTP Results:

There was no difference in LTP between control and sarin-treated groups. Note, however, that there was a large amount of variability in the responses during the potentiated responses. Although this was the case in slices from control and sarin-treated rats, there was somewhat more variability in the treated rats. It is possible that the presence of chronic seizure activity in some of the treated rats could have contributed to the variability (as suggested by Armstrong, et al., 1997). However, there also was appreciable variability in the control potentiated responses. Some of that variability simply may be due to the difficulty in doing *in vitro* LTP in adult animals. This is not necessarily surprising, because adult brain tissue is less tolerant of the trauma of slicing, etc., than the tissue from much younger (P14-30) rats typically used for LTP studies.

Methods for the High Potassium ACSF Model (High K⁺-ACSF):

Animals were injected and allowed to recover as described in the section for the LTP experiments. Brain slices were obtained as described for all experiments for which both LTP and High K⁺-ACSF models were used on a given pair of rats (n=9 pairs). Before LTP experiments were begun, 15 pairs of rats (30 total) were used for a range of High K⁺-ACSF experiments. In those experiments, the brain slices (625 μ m) were cut using a McIlwain tissue chopper after the hippocampus was dissected out of the brain. In the experiments in which

Mcllwain tissue chopper slices were used, the ACSF composition was as follows: 120mM NaCl; 3.3 mM KCl, 1.23 mM NaPO₄, 25 mM NaHCO₃, 0.9mM Mg SO₄, 1.3 mM CaCl₂, 10mM dextrose, except during the room-temperature 1-hour recovery period, magnesium was elevated to 4.0 mM to promote recovery.

After the recovery period, one slice from each pair was placed in the recording chamber so that both slices were exposed to identical conditions during the same post-dissection time. Recording and stimulating electrodes were similar to those used in the LTP experiments. The recording electrode were placed in *stratum pyramidale* of hippocampal area CA3 and stimulating electrodes were placed in the Schaffer collateral pathway in area CA3 at the level of the fimbria. Waveforms of extracellularly recorded evoked responses were saved on a Nicolet 430 oscilloscope for later analysis and continuous activity was recorded on videotapes using a VCR (Toshiba/Unitrade, PCM recording) and captured live on a Gould 220 chart recorder.

Baseline extracellular responses were delivered at 30-60-second intervals for a 20 minute period. Then an I/O curve from 100-600 μ A was obtained to assess the responses of the slices in normal ACSF. After the I/O curve, a 30 minute stimulus-free period was recorded while the slice was still exposed to control ACSF. This assesses the presence of any spontaneous epileptiform bursts (sEBs). No stimulus pulses are given during this period because the spontaneous occurrence of sEBs can be occluded by repeated stimuli (such as those given during the baseline and I/O curve). Next, an ACSF solution containing elevated potassium (5 mM) was bath-applied for 30 minutes. This recording period (as well as the subsequent high potassium and “control ACSF wash” exposures), were also stimulus-free, to avoid disrupting or triggering the occurrence of any spontaneous activity. Next, a 30 minute “control ACSF wash” period was done in some slices to assess the recovery of the slice from the 5 mM KCl ACSF solution. This

was followed by a 7 mM KCl ACSF solution, which is also followed by another “control ACSF wash” period. In some slices, the 5 mM KCl ACSF was followed directly by the 7 mM KCl solution. If spontaneous activity arose in the control or 5 mM potassium ACSF, this result suggests that the slice may be abnormally hyperexcitable. These sEBs are from 1-3 mV in magnitude and range from 50-250 ms in duration and are clearly visible on the chart recorder tracings, since they are well above the baseline noise. Any events that were questionable were assessed by playing the VCR recording back through the oscilloscope. (Static or other types of electronic noise are easily detected by this method and omitted.) The sEBs were counted on the chart recorder paper during the last 10 minutes of each exposure time.

High Potassium Results:

In our pilot studies in which McIlwain slices were used, when slices were first exposed to control ACSF, sarin-exposed rats appeared to be more hyperexcitable than slices from saline-exposed rats - at least in several pairs. However, often, both slices showed some level of hyperexcitability (such as, more than one orthodromic population spike following a stimulus pulse). Thus, although some pairs seemed to suggest a difference, this finding was not consistent. Once again, this variability could be a function of the age of the animals used, because in young animals (in which this type of study is most often done) there are seldom signs of hyperexcitability in normal slices exposed to control ACSF. One possibility was that the McIlwain tissue chopper method was too drastic for the adult slices, so we switched to vibratome slices for subsequent studies, since that slicing method is considered to be more gentle.

In the subsequent studies in which vibratome slices were used, there was also not a striking difference between slices from sarin- and saline-exposed rats. As shown below, neither the number of slices with sEBs nor the average number of sEBs/slice in each solution were strikingly different. There was only one measure in which there was any difference (the number of slices exposed to KCl 7 mM that had any sEBs in saline-exposed rats was 4 of 7 slices, whereas in sarin-exposed rats it was 7 of 7). However, the fact that in both sarin and control groups, 2 of 7 slices had sEBs even in control ACSF (the nl ACSF column) suggested that this method was too disruptive for the tissue from adult rats, because normal, healthy tissue should not be this hyperexcitable.

Thus, two different experimental approaches did not show any clear significant electrophysiological abnormalities following sarin exposure at time periods tested in our studies. Further detailed analyses using better technologies are required to delineate electrophysiological abnormalities due to sarin exposure.

Table 1: Results from the vibratome-cut slices exposed to normal ACSF, followed by 5 mM and 7 mM KCl ACSF (with or without a wash phase between the two solutions). Results from slices from control animals are reported first, then the sarin-exposed animals are in the lower columns. The numbers in parenthesis are the number of slices for this measure.

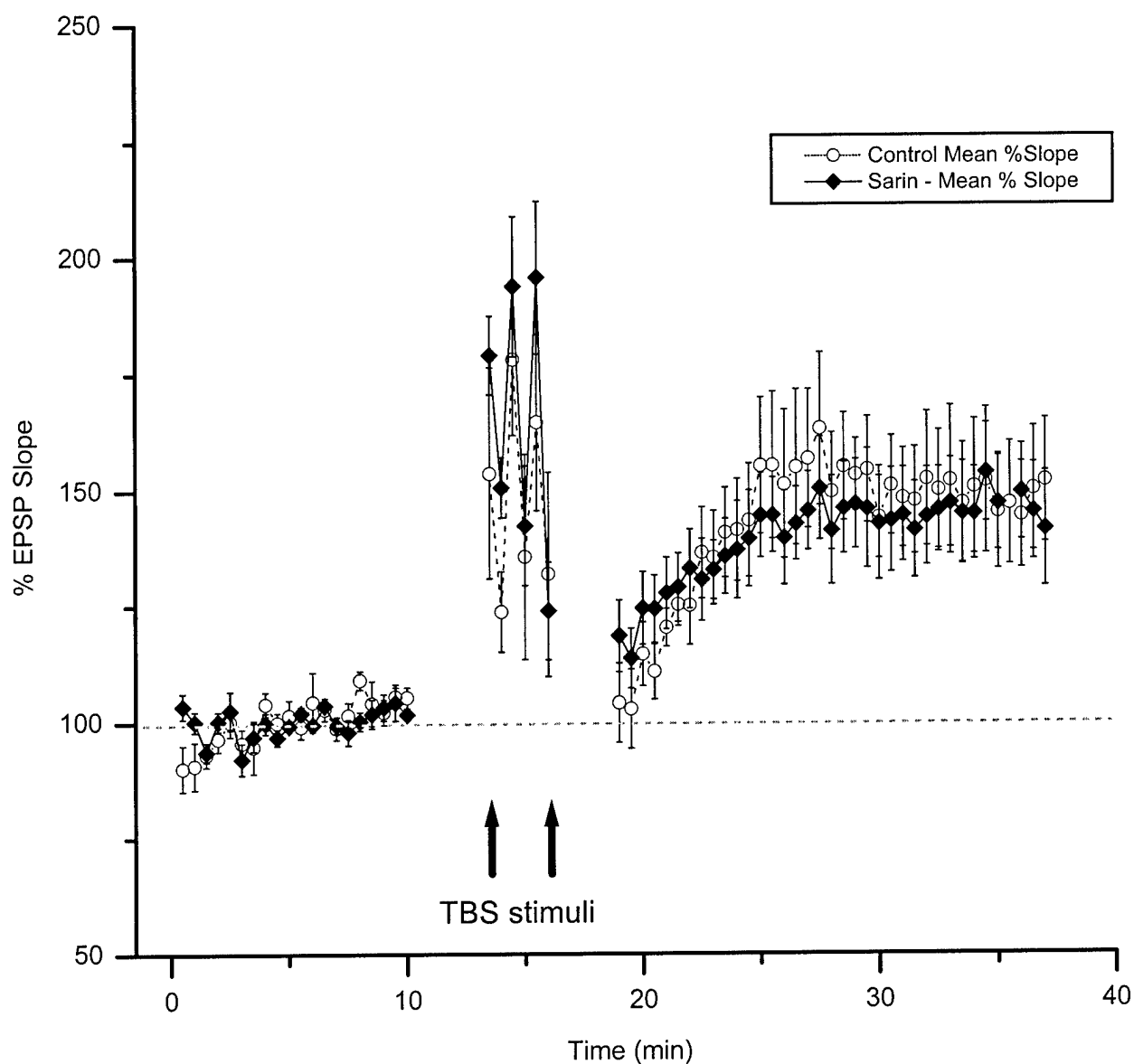
CONTROL	nl ACSF	KCl 5 mM	wash ACSF	KCl 7 mM	wash ACSF
#slices with sEB	2 of 7	3 of 7	1 of 2	4 of 7	3 of 5
Ave sEBs (n)	8 (2)	29.3 (3)	7 (1)	77.5 (4)	8.67 (3)
range	6 to 10	9 to 41	0-7	47-109	2 to 18
Sarin	nl ACSF	KCl 5 mM	wash (+/_)	KCl 7 mM	wash
#slices sEB:total	2 of 7	6 of 7	1 of 2	7 of 7	2 of 4
Ave sEBs	20.5 (2)	36.5 (6)	10 (1)	64.57 (7)	10.5 (2)
range	10 to 31 (2)	7 to 78 (6)	10 (1)	16 to-144 (7)	3 to 18 (2)

nl ACSF = normal ACSF

slices with sEBs = the number of slices that had any sEBs at all.

Ave sEBs = the number of sEBs that occurred during the last 10 minute period of each solution.

range: range of sEB number for all slices (see previous row for number of slices)



Long-Term Potentiation (LTP) in control (open circles) and sarin-exposed (LD50) (black diamonds) rats. A 10 minute baseline recording period is followed by the responses recorded during the theta-burst stimuli TBS (arrows). After the TBS stimuli, the subsequent potentiated responses are recorded for 30 minutes. Responses are normalized to baseline and reported as %EPSP slope \pm SEM. (n=9 animals per group) The dotted line approximates the baseline (100%) level, to facilitate comparison at the later time points.

Specific Aim 2. Effect of stress on sub-clinical sarin exposure.

Rationale: We have been testing the hypothesis that interactions between low-dose sarin and exposure to combat environment stress would result in exacerbated toxicological effects than sarin alone. Stress is common mechanism to cope with an unusual circumstance; the stress response may help in avoiding an unusual situation or may result in exacerbated outcome. It has been recognized that deployed personnel in an armed conflict do experience certain degree of stress, which in some cases may lead to severe health problems, such as post-traumatic stress syndrome. During PGW, some military personnel did experience certain degree of stress (Baker et al. 1997). Thus, it is possible that exposure to subclinical levels of sarin in combination with stress may lead to neurotoxicological effects that may not be observed with exposure to low-levels of sarin alone.

Materials and Methods: Male Sprague-Dawley rats (225-250g) were obtained from Zivic-Miller Laboratories, Allison Park, PA. The animals were housed in Duke University Medical Center vivarium at 21-23 °C and on a 12-h dark-light cycle. The animals were allowed food and water *ad libitum*. All the treatments and procedures on the animals were carried out strictly according to the recommended guidelines by the Duke University Institutional Animal Care and Use Committee and U.S. Department of Defense.

Treatment of Animals and Tissue Retrieval

Animals were randomly divided into following groups of 5 animals each for each time point of the study.

Control: The rats in this group were handled without subjecting them to the stress daily for 15 days, and then on the last day treated with single i.m. injection of saline.

Stress alone: The rats were subjected to daily immobilization stress by placing them in Plexiglas restrainer tube for 5 minutes, daily for 15 days.

sarin alone: The rats were treated with single i.m. injection of 0.5xLD50 sarin.

Co-exposure to stress and sarin: The rats were subjected to daily immobilization stress by placing them in Plexiglas restrainer tube for 5 minutes, daily for 15 days. On day 15 following completion of the stress, the rats were treated with single i.m. injection of 0.5xLD50 sarin.

At 24 hrs, 3 and 6 month following treatment with sarin, the rats were anesthetized with ketamine/xylazine (100mg/kg ketamine, 15mg/kg xylazine) and blood was drawn in heparinized syringe. Brains were removed and washed thoroughly with ice cold normal saline to remove blood. Brain regions, cortex, midbrain, cerebellum and brainstem were dissected on ice and snap frozen in liquid nitrogen. Plasma was separated and frozen at -80 °C for enzyme studies.

Behavioral Testing Battery:

The behavioral tests employed in these studies evaluate sensorimotor reflexes, motor strength, and coordination. All behavioral testing was performed by an observer blind to the animal's treatment status and was carried out in a sound-proof room with subdued lighting (less than 10.76 lumens/m², ambient light) between 7 and 11:30 AM. Behavioral testing was carried out at 3 and 6 months following single i.m. injection of 0.5xLD50 sarin.

Reflexes:

Postural reflexes (Bederson, et al,1986; Markgraf, et al,1992), visual, tactile and proprioceptive forelimb placing responses (Markgraf et al, 1992) and orienting to

vibrissae touch (Whishaw et al, 1985) were carried out as described by Abou-Donia et al (2002).

Inclined Plane:

Description

Rats were placed on a flat plane in the horizontal position, with the head facing the side of the board to be raised (Yonemori et al, 1998) and Abou-Donia et al (2002).

Scoring: The angle that the rat begins to slip downward was recorded. The results of the two trials were averaged for each testing session.

Forepaw Grip Time:

Description

The rat's forepaw strength was assessed by having them grip a 5 mm diameter wood dowel that was held horizontally and raised so that the rat supports its body weight as described by Andersen et al (1991) and Abou-Donia et al (2002).

Scoring: Time to release grip was recorded in seconds. The results of the two trials were averaged for each testing session.

Beam-Walking:

Description

The testing apparatus was a 2.5 X 122 cm wooden beam elevated 75.5 cm above the floor with wooden supports as described by Goldstein (1993) and Abou-Donia et al (2002).

Scoring: Beam-walking ability was measured with a seven point scoring system scale as previously described by Goldstein (1993 & 1995) as detailed by Abou-Donia et al (2002).

Statistical Analyses

Comparisons across treatment groups for postural reflexes, limb placing, and vibrissae touch orientation were analyzed with non-parametric analysis of variance (Kruskal-Wallis test). Data for the remaining behavioral tests were compared among groups by one-way or two-way repeated measures ANOVA as appropriate. If a significant difference was found, Fisher's LSD tests were applied to permit post-hoc, pair-wise comparisons. A two-tailed P value of <0.05 was considered statistically significant.

Cholinesterase determination:

Activities of AChE in brain regions and BChE in plasma were determined according to the method of Ellman et al, (1961), modified for assay in a Molecular Devices UV Max Kinetic Microplate Reader, as previously described (Abou-Donia et al, 1996, 2001, 2002). Protein concentration was determined by BCA method according to Smith et al, (1985). The enzyme activities are expressed as μ mole substrate hydrolyzed/min/mg protein.

Statistical Analysis:

The results were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test. P value <0.05 was considered significant.

Results and conclusions: Neurobehavioral evaluations for sensorimotor performance are presented in Fig 1 A& B for 3 and 6 months, respectively. With the exception of beam-walk scores, each treatment resulted in sensorimotor impairments as reflected on each test and at both the time points. However, there was no additive effect when the rats were exposed to sarin and stress in combination. For beam-walk time, incline plane and

forepaw grip time, there were significant effects following each treatment on 3 and 6 months time points as compared to controls.

Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) are presented in Fig (2–4) for 24 hrs, 3 months and 6 months respectively. Treatment with sarin alone did not cause any significant in brain regional or plasma cholinesterase activity except that in the cortex, where there was a nonsignificant decrease (~76% of control) at 24 hrs after the sarin treatment. Treatment with stress alone, on the other hand, caused a significant increase in brainstem (~138% of control) and midbrain (~127% of control) AChE activity. Treatment with the combination of sarin and stress did not result in any significant change. Brain regional AChE and plasma BChE activity from 3 month group of rats are presented in Fig 3. There was a significant increase in cortex AChE activity (~159% of control) following treatment with sarin alone. A significant increase in cortex AChE activity (~202% of control) was observed in the rats treated with stress alone. Figure 4 demonstrates the brain regional AChE and plasma BChE activity 6 months after sarin treatment. There was a significant increase (~124% of control) in cerebellum AChE activity in the rats treated with sarin alone. Stress exposure alone resulted in a significant increase in midbrain and cerebellum AChE activity (~128% of control). Treatment with sarin and stress in combination caused a significant decrease in midbrain AChE activity (~59% of control).

Figure Legends:

Figure 1: Sensorimotor performance following a single i.m. treatment with 0.5x LD50 sarin, alone and in combination with immobilization stress. **A:** After 3 month of sarin treatment; **B:** After 6 month of sarin treatment. The rats were subjected to immobilization stress for 5 minutes by placing them in a Plexiglas restrainer for 15 days. The control and sarin group rats were handled similarly, but without restrainer. On day 15, following the last exposure to stress, the rats were treated with a single i.m. injection of 0.5xLD50 sarin. The rats from control and stress alone group were treated with i.m. injection of normal saline. The rats were maintained for a further period of 3 and 6 months. The animals were evaluated for beam-walk score, beam-walk time, incline plane and grip response. The data are presented as mean \pm SE, n=5. *Indicates statistically significant.

Figure 2: Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) activity 24 hrs after single i.m. treatment with 0.5x LD50 sarin. **A:** AChE activity in different brain regions; **B:** BChE activity in plasma. The rats were subjected to immobilization stress for 5 minutes by placing them in a Plexiglas restrainer for 15 days. The control and sarin group rats were handled similarly, but without restrainer. On day 15, following the last exposure to stress, the rats were treated with a single i.m. injection of 0.5xLD50 sarin. The rats from control and stress alone group were treated with i.m. injection of normal saline. The rats were sacrificed 24 hrs after the treatment with sarin. Enzyme activity was determined as described in Materials and Methods. The control activity was 0.79 ± 0.11 cortex; 1.39 ± 0.06 brainstem; 1.39 ± 0.06 midbrain; 0.73 ± 0.04 cerebellum and 0.03 ± 0.001 , plasma expressed as μ moles

substrate hydrolyzed/min/mg protein. The data are presented as mean + SE (% of control), n = 5. * Indicates statistically significant.

Figure 3: Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) 3 months after a single i.m. treatment with 0.5x LD50 sarin. **A:** AChE activity in different brain regions; **B:** BChE activity in plasma. The rats were subjected to immobilization stress for 5 minutes by placing them in a Plexiglas restrainer for 15 days. The control and sarin group rats were handled similarly, but without restrainer. On day 15, following the last exposure to stress, the rats were treated with a single i.m. injection of 0.5xLD50 sarin. The rats from control and stress alone group were treated with i.m. injection of normal saline. The rats were maintained for a further period of 3 months. Enzyme activity was determined as described in Materials and Methods. The control activity was 0.88 ± 0.06 cortex; 2.01 ± 0.16 brainstem; 1.38 ± 0.08 midbrain; 0.48 ± 0.02 cerebellum and 0.039 ± 0.001 , plasma expressed as μ moles substrate hydrolyzed/min/mg protein. The data are presented as mean + SE (% of control), n = 5. * Indicates statistically significant.

Figure 4: Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) 6 months after a single i.m. treatment with 0.5x LD50 sarin. **A:** AChE activity in different brain regions; **B:** BChE activity in plasma. The rats were subjected to immobilization stress for 5 minutes by placing them in a Plexiglas restrainer for 15 days. The control and sarin group rats were handled similarly, but without restrainer. On day 15, following the last exposure to stress, the rats were treated with a single i.m. injection of 0.5xLD50 sarin. The rats from control and stress alone group were treated with i.m. injection of normal saline. The rats were maintained for a

further period of 6 months. Enzyme activity was determined as described in Materials and Methods. The control activity was 1.27 ± 0.28 cortex; 1.48 ± 0.09 brainstem; 1.20 ± 0.05 midbrain; 0.5 ± 0.02 cerebellum and 0.06 ± 0.01 , plasma expressed as μ moles substrate hydrolyzed/min/mg protein. The data are presented as mean + SE (% of control), n = 5. * Indicates statistically significant.

Table 1

ChE Activity (stress/sarin-24hr)
mean value (μ moles/min/mg protein)

Regions	name of the group							
	control		STRESS		SARIN		STRESS / SARIN	
	mean	SE	mean	SE	mean	SE	mean	SE
Cortex	0.79	0.11	0.91	0.12	0.6	0.13	0.72	0.07
Brainstem	1.39	0.21	1.92 *	0.06	1.88	0.1	1.3	0.21
Midbrain	1.39	0.06	1.77 *	0.09	1.56	0.08	1.56	0.06
Cerebellum	0.73	0.04	0.79	0.06	0.72	0.07	0.71	0.03
Plasma	0.03	0.001	0.03	0.01	0.03	0.001	0.03	0.01

ChE Activity (stress/sarin-24hr)
(% of control)

Regions	name of the group							
	control		STRESS		SARIN		STRESS / SARIN	
	% of control	SE	% of control	SE	% of control	SE	% of control	SE
Cortex	100	13.4	115.6	15.1	76.7	15.9	91.1	9.1
Brainstem	100	14.9	138.2 *	4.6	135.5	7.4	93.6	15.2
Midbrain	100	4	127.3 *	6.8	112.7	6.1	112.7	4.4
Cerebellum	100	5.9	107.9	8	98.7	10.1	96.2	3.6
Plasma	100	16.5	104.1	21.7	113.3	14.4	113.7	19.1

Table 2

ChE Activity (stress/sarin-3 months)
mean value (μ moles/min/mg protein)

Regions	name of the group							
	control		STRESS		SARIN		STRESS / SARIN	
	mean	SE	mean	SE	mean	SE	mean	SE
Cortex	0.88	0.065	1.78 *	0.46	1.4 *	0.24	0.94	0.16
Brainstem	2.01	0.16	2.65	0.58	1.61	0.18	2.13	0.2
Midbrain	1.38	0.08	1.59	0.08	1.4	0.1	1.44	0.05
Cerebellum	0.48	0.02	0.5	0.037	0.48	0.3	0.49	0.03
Plasma	0.039	0.001	0.031	0.005	0.038	0.01	0.039	0.01

ChE Activity (stress/sarin-3 months)
(% of control)

Regions	name of the group							
	control		STRESS		SARIN		STRESS / SARIN	
	% of control	SE	% of control	SE	% of control	SE	% of control	SE
Cortex	100	7.4	202.5 *	52.9	159.9 *	27.4	107.1	18.6
Brainstem	100	8	104.7	12.8	79.9	8.7	106.1	9.7
Midbrain	100	6	115.9	5.9	102.6	7.3	105.4	3.7
Cerebellum	100	3.9	102.8	7.8	100	6.5	101.7	5.4
Plasma	100	3.4	79.22	20.6	98.2	20.1	101.2	21.2

Table 3

ChE Activity (stress/sarin-6months)
mean value (μ moles/min/mg protein)

Regions	name of the group							
	control		STRESS		SARIN		STRESS / SARIN	
	mean	SE	mean	SE	mean	SE	mean	SE
Cortex	1.27	0.28	0.84	0.23	1.33	0.43	1.26	0.37
Brainstem	1.48	0.09	1.21	0.19	1.48	0.07	1.6	0.13
Midbrain	1.2	0.05	1.52 *	0.09	1.59	0.27	0.707 *	0.01
Cerebellum	0.5	0.02	0.65 *	0.04	0.62 *	0.03	0.7 *	0.03
Plasma	0.06	0.01	0.06	0.01	0.07	0.01	0.06	0.004

ChE Activity (stress/sarin-6months)
(% of control)

name of the group								
Regions	control		STRESS		SARIN		STRESS / SARIN	
	% of control	SE	% of control	SE	% of control	SE	% of control	SE
Cortex	100	22.4	66.7	18.1	105.2	34.6	99.1	29.7
Brainstem	100	6.3	81.9	12.6	99.6	4.9	107.9	8.5
Midbrain	100	4.3	128.1 *	7.5	134.7	22.6	59.6 *	0.4
Cerebellum	100	4.3	130.7 *	7.9	124.8 *	6.5	141.6 *	5.2
Plasma	100	14.4	101.3	19.5	117.9	9.4	101.9	6.2

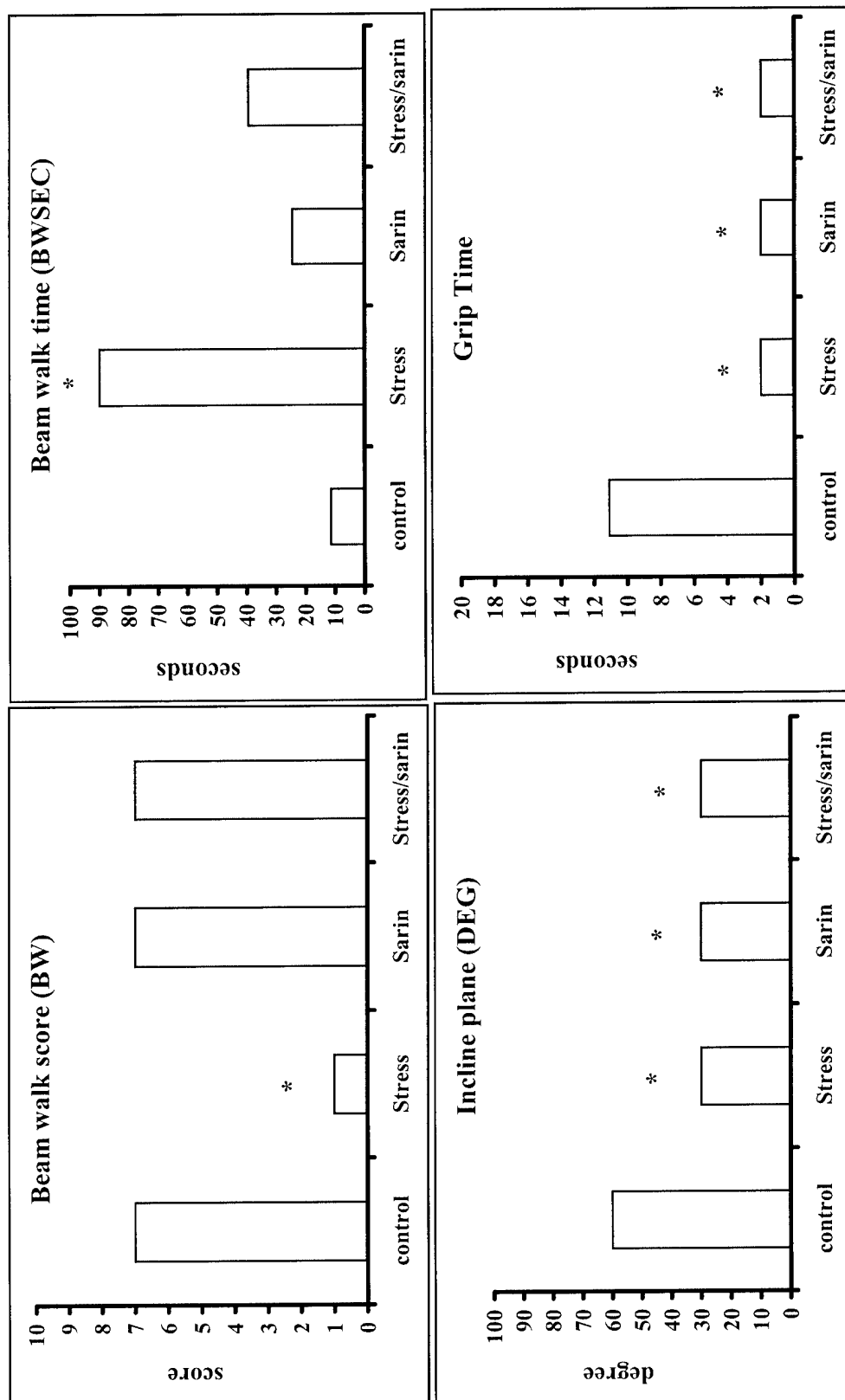


Figure 1 A : Behavioral studies (3 months)

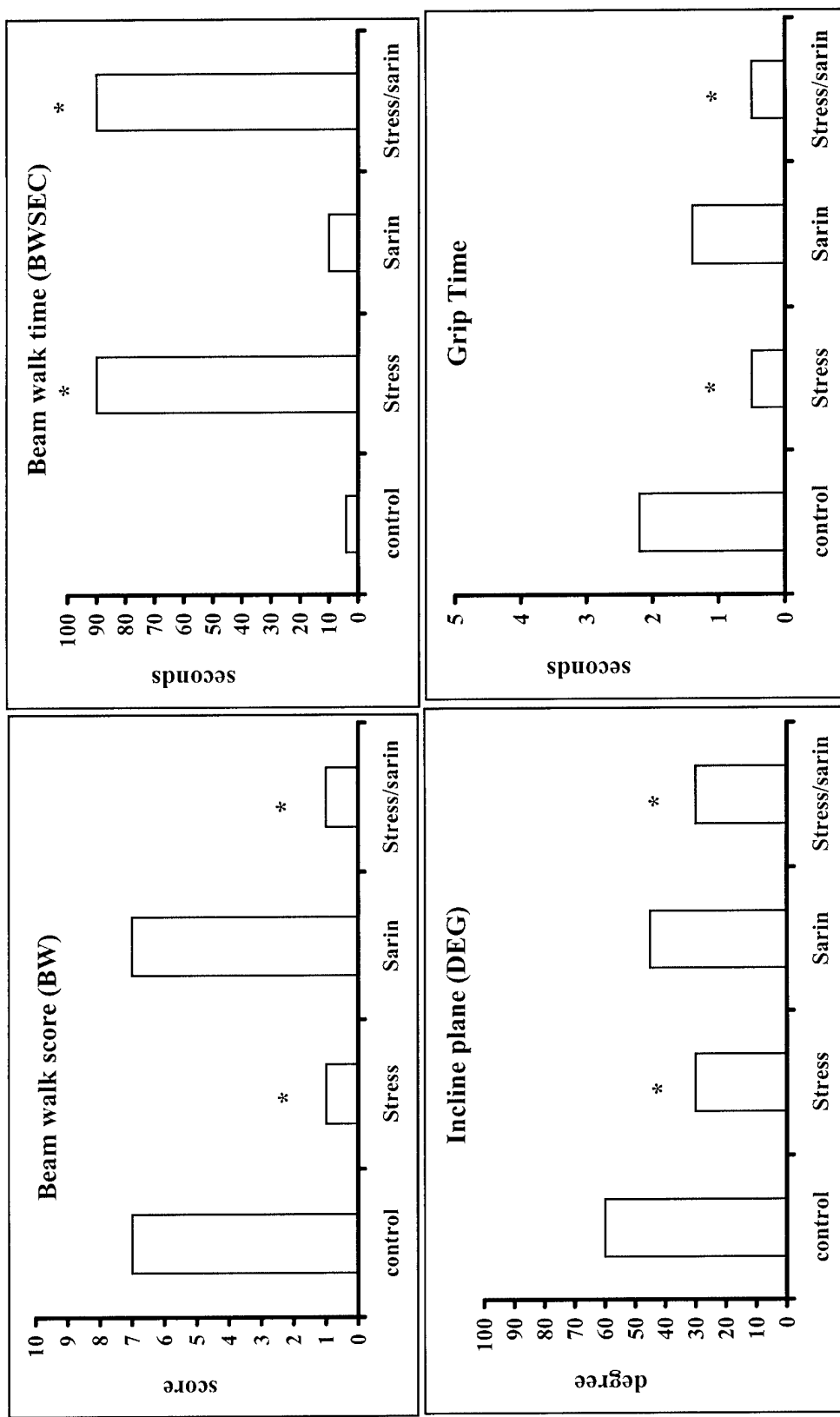


Figure 1 B : Behavioral studies (6 months)

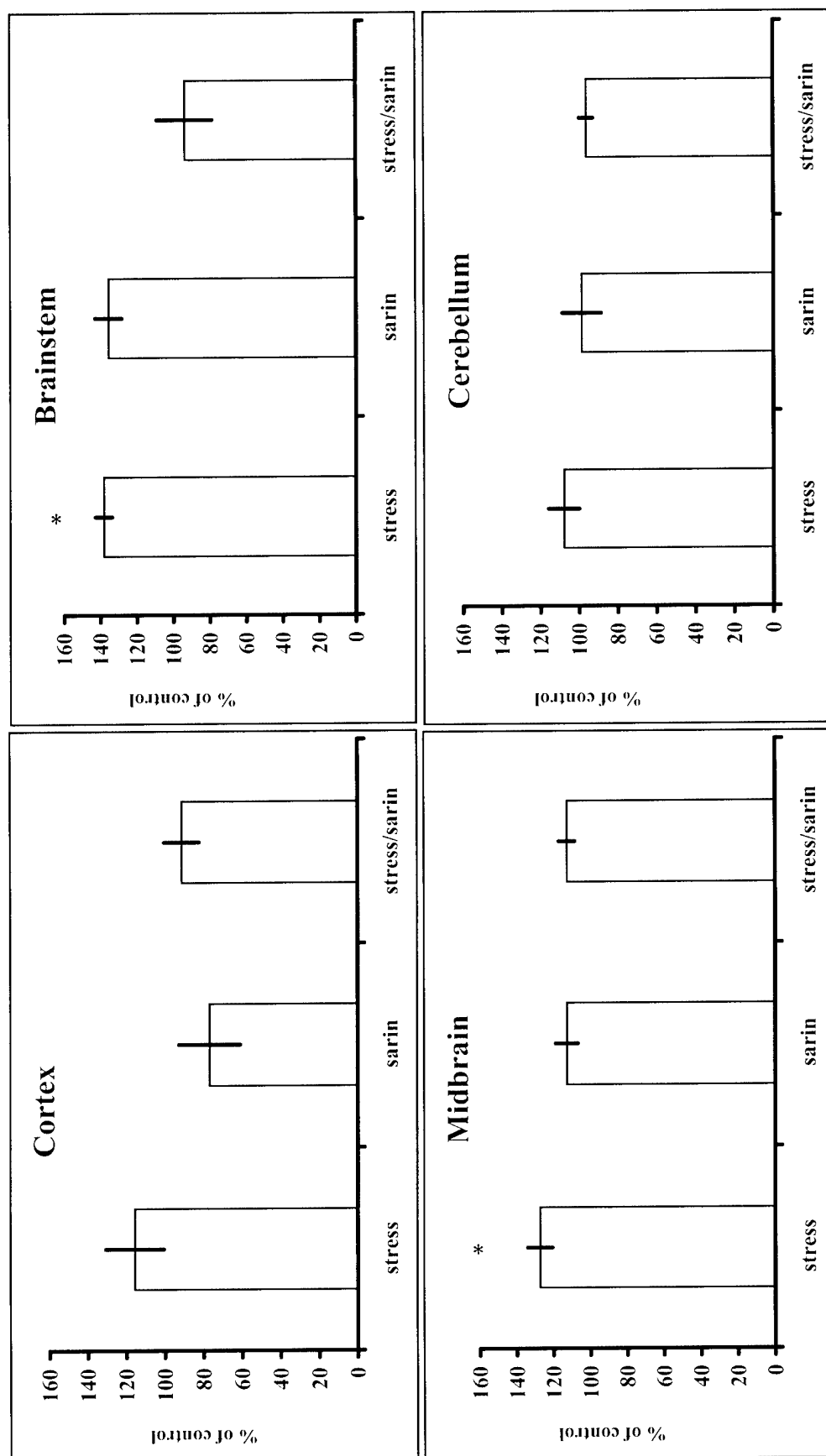


Figure 2 A : Brain regional AChE activity (Stress/sarin-24hr)

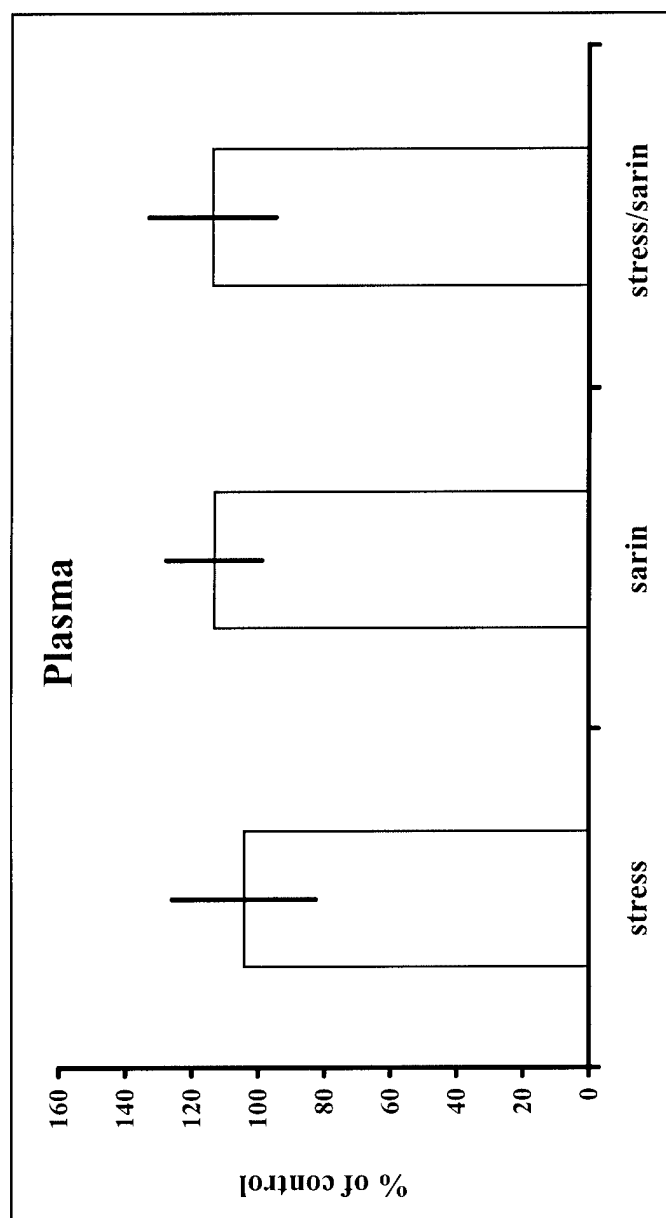


Figure 2 B : Plasma BChE activity (Stress/sarin-24hr)

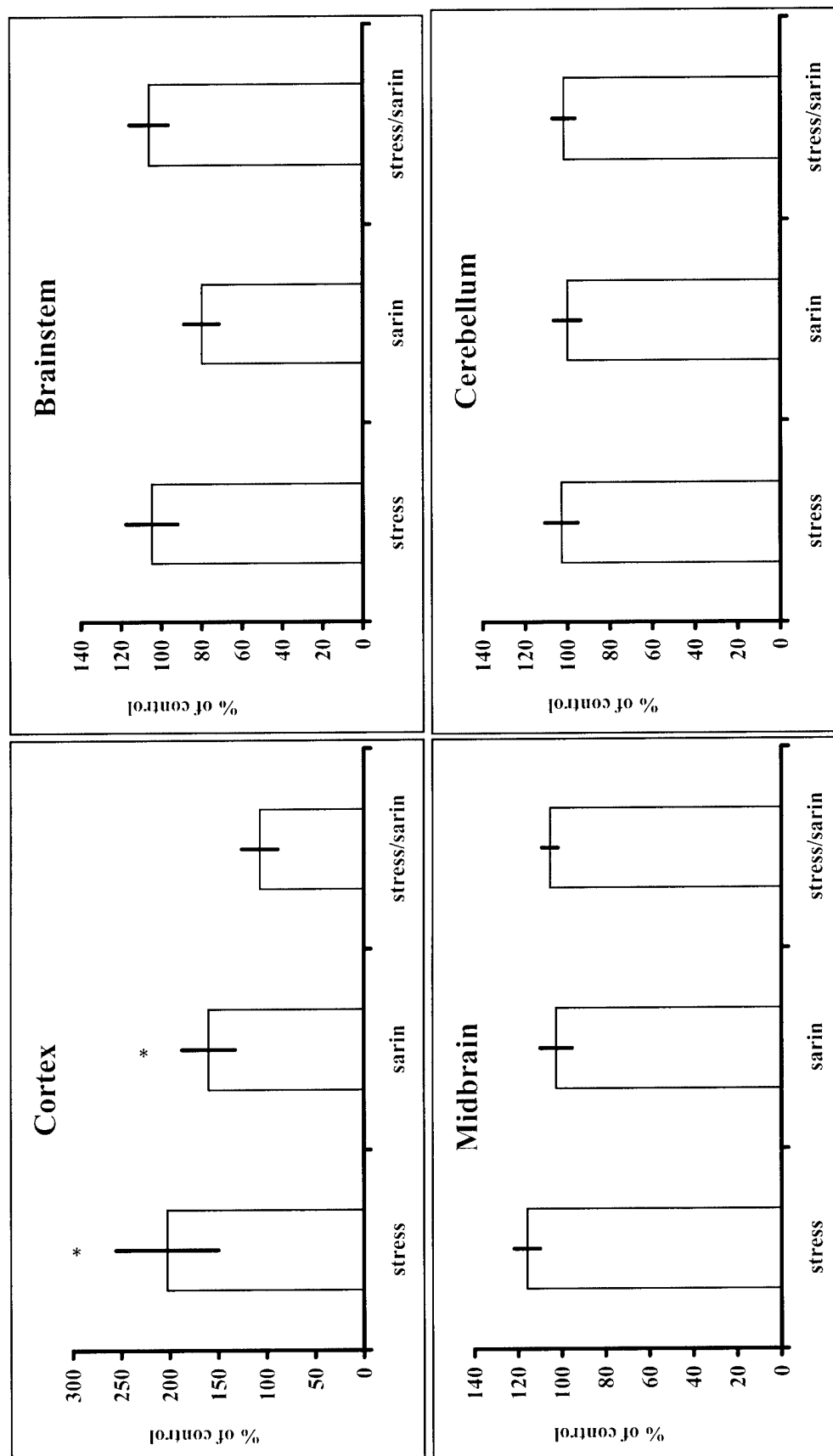


Figure 3 A : Brain regional AChE activity (Stress/sarin-3 months)

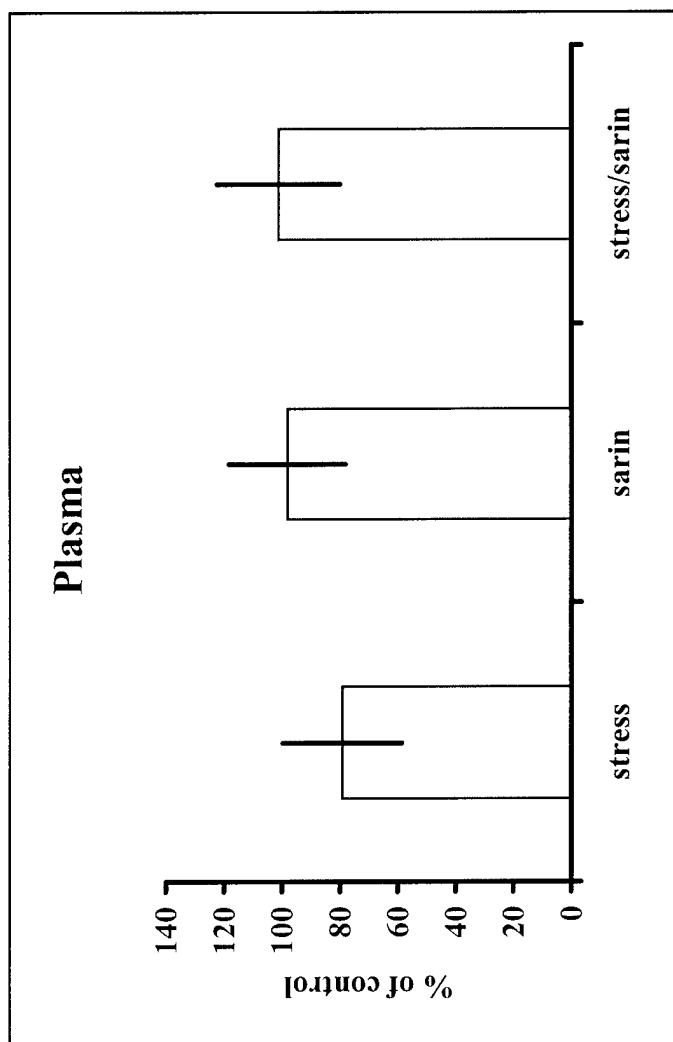


Figure 3 B : Plasma BChE activity (Stress/sarin-3 months)

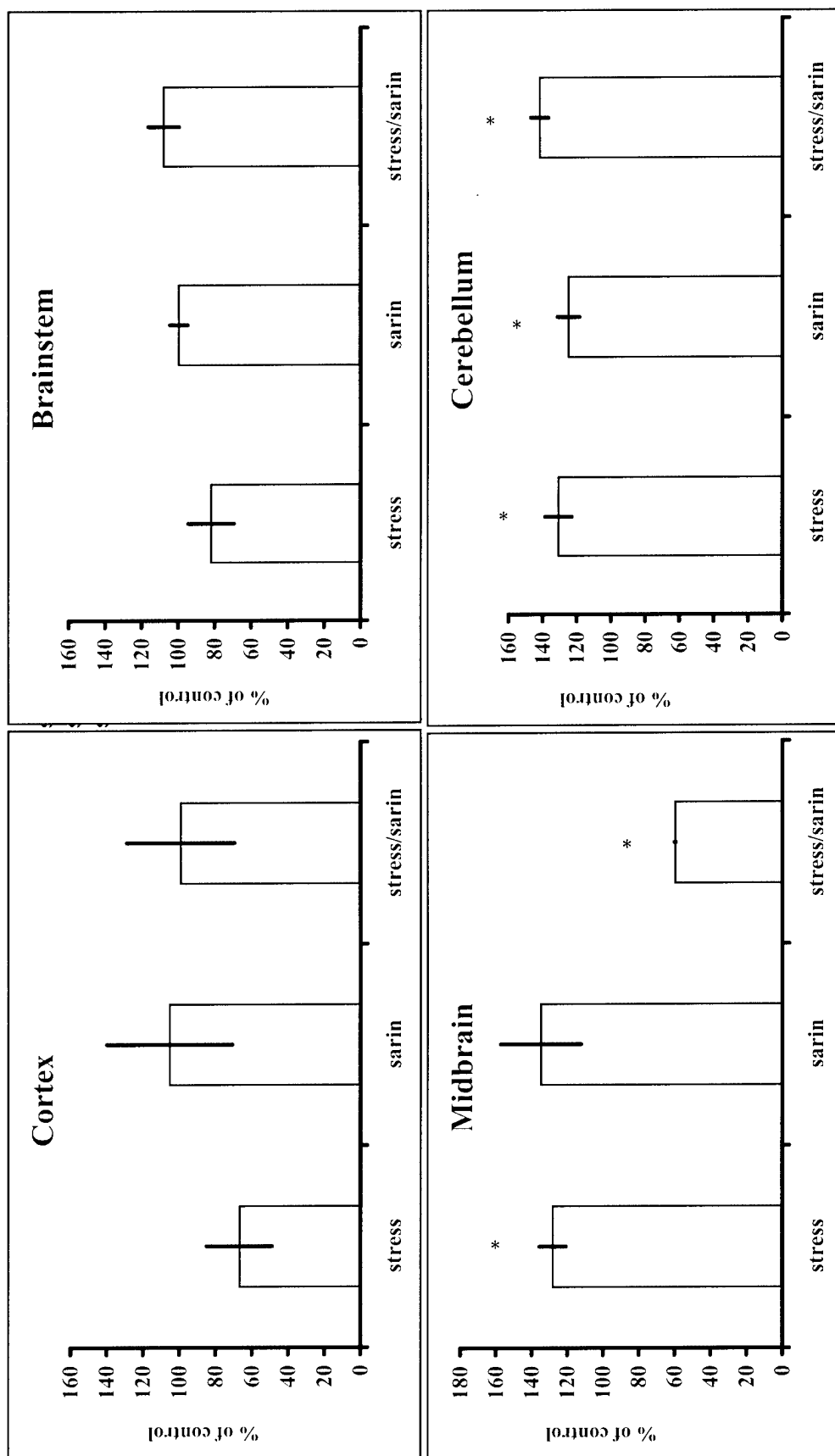


Figure 4 A : Brain regional AChE activity (Stress/sarin-6months)

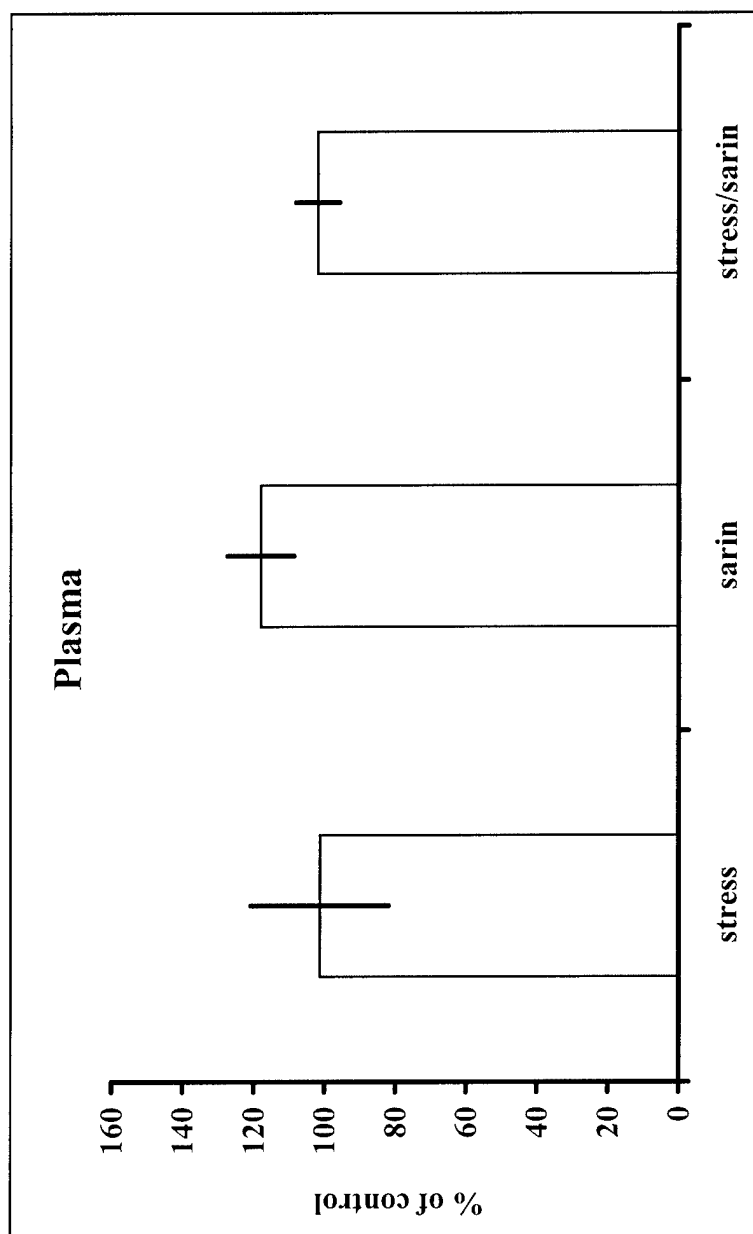


Figure 4 B : Plasma BChE activity (Stress/sarin-6months)

Specific Aim 3. Effect of heat on sub-clinical sarin exposure.

Rationale: The deployed personnel in PGW had to face extreme conditions of weather such as day time heat. Excessive exposure to heat causes central nervous system abnormalities (Sharma and Day, 1987). We have been testing the possibility that interactions between low-dose sarin and exposure to the prevailing environmental factors such as heat would result in greater toxicological manifestations than sarin alone.

Materials and Methods: Male Sprague-Dawley rats (225-250g) were obtained from Zivic-Miller Laboratories, Allison Park, PA. The animals were housed in Duke University Medical Center vivarium at 21-23 °C and on a 12-h dark-light cycle. The animals were allowed food and water *ad libitum*. All the treatments and procedures on the animals were carried out strictly according to the recommended guidelines by the Duke University Institutional Animal Care and Use Committee and U.S. Department of Defense.

Treatment of Animals and Tissue Retrieval

Animals were randomly divided into following groups of 5 animals each for each time point of the study.

Control: The rats were placed in the incubator for 4 hrs at room temperature and then treated with single i.m. injection of saline.

Heat alone: The rats were placed in a biological incubator at 38°C for 4 hrs.

sarin alone: The rats were treated with single i.m. injection of 0.5xLD50 sarin.

Co-exposure to heat and sarin: The rats were placed in a biological incubator at 38°C for 4 hrs. Immediately following the heat treatment, the rats were treated with single i.m. injection of 0.5xLD50 sarin.

At 3 hrs, 3 and 6 month following treatment with sarin, the rats were anesthetized with ketamine/xylazine (100mg/kg ketamine, 15mg/kg xylazine) and blood was drawn in heparinized syringe. Brains were removed and washed thoroughly with ice cold normal saline to remove blood. Brain regions, cortex, midbrain, cerebellum and brainstem were dissected on ice and snap frozen in liquid nitrogen. Plasma was separated and frozen at -80 °C for enzyme studies.

Behavioral Testing Battery:

The behavioral tests employed in these studies evaluate sensorimotor reflexes, motor strength, and coordination. All behavioral testing was performed by an observer blind to the animal's treatment status and was carried out in a sound-proof room with subdued lighting (less than 10.76 lumens/m², ambient light) between 7 and 11:30 AM. Behavioral testing was carried out at 3 and 6 months following single i.m. injection of 0.5xLD₅₀ sarin as described in specific aim 2.

Statistical Analyses

Comparisons across treatment groups for postural reflexes, limb placing, and vibrissae touch orientation were analyzed with non-parametric analysis of variance (Kruskal-Wallis test). Data for the remaining behavioral tests were compared among groups by one-way or two-way repeated measures ANOVA as appropriate. If a significant difference was found, Fisher's LSD tests were applied to permit post-hoc, pair-wise comparisons. A two-tailed P value of <0.05 was considered statistically significant.

Cholinesterase determination:

Activities of AChE in brain regions and BChE in plasma were determined in a Molecular Devices UV Max Kinetic Microplate Reader, as described in specific aim 2. The enzyme activities are expressed as μ mole substrate hydrolyzed/min/mg protein.

Blood-brain-barrier (BBB) Permeability: For the evaluation of BBB permeability changes, the rats were treated with 1.3 mg/kg PB (oral by gavage) containing 100 μ Ci [3 H]PB during the last 30 minutes of 4 hrs of heat stress. The animals were perfused for 15 minutes by transcardial perfusion 3 hrs after single i.m. injection with 0.5xLD50 sarin. The brain regions were dissected and the radioactivity in each brain region was analyzed following solubilization of the tissue.

Statistical Analysis:

The results were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test. P value <0.05 was considered significant.

Results and conclusions: The data are presented in appendix 3. Neurobehavioral evaluations for sensorimotor performance are presented in Fig 1 A& B for 3 and 6 months, respectively. Each treatment resulted in sensorimotor impairments in each test, and at 3 and 6 months following sarin treatment. However, there was no additive effect when the rats were exposed to sarin and heat in combination. For incline plane, the rats treated with heat and sarin did not any further deterioration. In fact, at 3 month time point, the rats exposed to heat and sarin showed improvement in incline plane response. All the parameters showed a worsening response over 6 month period following treatment with sarin.

Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) are presented in Fig (2–4) for 3 hrs, 3 months and 6

months, respectively. Treatment with sarin alone caused a significant decrease (40-63% of control) in brain regional acetylcholinesterase activity except that in the brainstem at 24 hrs after the sarin treatment. Treatment with heat alone, also caused a significant decrease in cortex (~61.8% of control) and midbrain (~39.4% of control) AChE activity and a significant increase (~253% of control) of plasma BChE activity. Treatment with the combination of sarin and heat did not result in any significant change than that caused by either heat or sarin alone. Brain regional AChE and plasma BChE activity from 3 month group of rats are presented in Fig 3. There was a significant increase in brainstem and cerebellum AChE activity (~120% of control) following treatment with heat alone and treatment with heat and sarin in combination (~ 122 & 130% of control). Figure 4 demonstrates the brain regional AChE and plasma BChE activity 6 months after sarin treatment. There was a significant increase (~115-147% of control) in brainstem, midbrain and cerebellum AChE activity in the rats treated with heat alone. Sarin exposure alone resulted in a significant increase in midbrain AChE activity (~144% of control). Treatment with heat and sarin in combination caused a significant increase in midbrain AChE and plasma BChE activity (~148% and 217% of control, respectively). These results suggest that exposure to heat, alone or in combination could cause long-term increases in the cholinesterase activity in midbrain that may result in neurological consequences.

Figure Legends:

Figure 1: Sensorimotor performance following a single i.m. treatment with 0.5x LD50 sarin, alone and in combination with heat stress. **A:** After 3 month of sarin treatment; **B:** After 6 month of sarin treatment. The rats were subjected to heat stress for 4 hrs by placing them in a biological incubator at 38°C. The control and sarin group rats were handled similarly, but without heat treatment. Following the exposure with heat, the rats were treated with a single i.m. injection of 0.5xLD50 sarin. The rats from control and stress alone group were treated with i.m. injection of normal saline. The rats were maintained for a further period of 3 and 6 months. The animals were evaluated for beam-walk score, beam-walk time, incline plane and grip response. The data are presented as mean \pm SE, n=5. *Indicates statistically significant.

Figure 2: Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) activity and blood-brain barrier (BBB) permeability 3 hrs after single i.m. treatment with 0.5x LD50 sarin, alone and in combination with heat. **A:** AChE activity in different brain regions; **B:** BChE activity in plasma; **C:** BBB permeability assessed by [³H]pyridostigmine bromide (PB) passage in the brain.

The rats were subjected to heat stress for 4 hrs by placing them in a biological incubator at 38°C. The control and sarin group rats were handled similarly, but without heat treatment. Following the exposure with heat, the rats were treated with a single i.m. injection of 0.5xLD50 sarin. The rats from control and heat alone group were treated with i.m. injection of normal saline. The rats were sacrificed 3 hrs after the treatment with sarin. Enzyme activity was determined as described in Materials and Methods. The control activity was 0.79 \pm 0.11 cortex; 1.39 \pm 0.06 brainstem; 1.39 \pm 0.06 midbrain; 0.73

± 0.04 cerebellum and 0.03 ± 0.001 , plasma expressed as μ moles substrate hydrolyzed/min/mg protein. The data are presented as mean + SE (% of control), n = 5.

* Indicates statistically significant.

For the studies on the passage of PB into the brain, the rats were treated with 1.3 mg/kg PB (oral by gavage) containing 100 μ Ci [3 H]PB during the last thirty minutes of 4 hrs of heat stress and the radioactivity in each brain region was analyzed following solubilization of the tissue. The data are presented as the mean + SE (% control) of the ratio of the radioactivity present in brain region and plasma.

Figure 3: Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) 3 months after a single i.m. treatment with 0.5x LD50 sarin, alone and in combination with heat. **A:** AChE activity in different brain regions; **B:** BChE activity in plasma. The rats were subjected to heat stress for 4 hrs by placing them in a biological incubator at 38⁰C. The control and sarin group rats were handled similarly, but without heat treatment. Following the exposure with heat, the rats were treated with a single i.m. injection of 0.5xLD50 sarin. The rats from control and heat alone group were treated with i.m. injection of normal saline. The rats were maintained for a further period of 3 months. Enzyme activity was determined as described in Materials and Methods. The control activity was 1.83 ± 0.42 cortex; 1.52 ± 0.08 brainstem; 1.79 ± 0.07 midbrain; 0.51 ± 0.03 cerebellum and 0.030 ± 0.005 , plasma expressed as μ moles substrate hydrolyzed/min/mg protein. The data are presented as mean + SE (% of control), n = 5. * Indicates statistically significant.

Figure 4: Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) 6 months after a single i.m. treatment with 0.5x LD50

sarin, alone and in combination with heat. **A:** AChE activity in different brain regions; **B:** BChE activity in plasma. The rats were subjected to heat stress for 4 hrs by placing them in a biological incubator at 38⁰C. The control and sarin group rats were handled similarly, but without heat treatment. Following the exposure with heat, the rats were treated with a single i.m. injection of 0.5xLD50 sarin. The rats from control and heat alone group were treated with i.m. injection of normal saline. The rats were maintained for a further period of 6 months. Enzyme activity was determined as described in Materials and Methods. The control activity was 1.57 \pm 0.32 cortex; 1.45 \pm 0.04 brainstem; 1.21 \pm 0.18 midbrain; 0.55 \pm 0.04 cerebellum and 0.08 \pm 0.01, plasma expressed as μ moles substrate hydrolyzed/min/mg protein. The data are presented as mean + SE (% of control), n = 5. * Indicates statistically significant.

Table 1A

ChE Activity (Heat/Sarin-24hr -experiment)
mean value (μ moles/min/mg protein)

Regions	name of the group							
	CONTROL		HEAT		SARIN		HEAT/SARIN	
	mean	SE	mean	SE	mean	SE	mean	SE
Cortex	1.47	0.16	0.91 *	0.08	0.59 *	0.11	0.78 *	0.17
Brainstem	2.38	0.09	2.66	0.05	2.62	0.34	2.83	0.19
Midbrain	1.57	0.05	0.62 *	0.13	0.86 *	0.09	0.72 *	0.12
Cerebellum	0.72	0.02	0.55 *	0.02	0.45 *	0.07	0.49 *	0.04
Plasma	0.02	0.003	0.05 *	0.01	0.009 *	0.001	0.02	0.003

ChE Activity (Heat/Sarin- 24hr-experiment)
(% of control)

Regions	name of the group							
	CONTROL		HEAT		SARIN		HEAT/SARIN	
	% of control	SE	% of control	SE	% of control	SE	% of control	SE
Cortex	100	10.96	61.8 *	5.64	40.1 *	7.7	53.3 *	11.67
Brainstem	100	3.85	111.8	2.04	110	14.07	118.7	8.16
Midbrain	100	2.87	39.4 *	8.94	54.5 *	5.7	45.6 *	7.86
Cerebellum	100	3.25	76.3 *	2.47	63.3 *	9.49	67.8 *	5.1
Plasma	100	13.14	253.7 *	22.91	46 *	9.02	117.6	17.14

Table 1 B

**BBB permeability changes (Heat/Sarin-24hr -experiment,
mean value (Ratio of [3H]PB -uptake (% control)**

Regions	name of the group							
	CONTROL		HEAT		SARIN		HEAT/SARIN	
	mean	SE	mean	SE	mean	SE	mean	SE
Cortex	0.15	0.01	0.2 *	0.01	0.21	0.03	0.2 *	0.01
Brainstem	0.11	0.01	0.18 *	0.02	0.18 *	0.02	0.13	0.01
Midbrain	0.14	0.01	0.21 *	0.02	0.18	0.02	0.17	0.02
Cerebellum	0.15	0.01	0.22	0.03	0.19	0.02	0.18	0.02

**BBB permeability changes (Heat/Sarin- 24hr-experiment,
(% of control)**

Regions	name of the group							
	CONTROL		HEAT		SARIN		HEAT/SARIN	
	% of control	SE	% of control	SE	% of control	SE	% of control	SE
Cortex	100	6.38	135.7 *	3.75	141.5	17.28	135.1 *	7.88
Brainstem	100	7.43	158.9 *	17.13	161.3 *	14.18	117.8	10.06
Midbrain	100	6.18	149.4 *	13.86	124.3	13.63	119.5	10.88
Cerebellum	100	9.06	146	17.14	126	15.66	122.5	15.79

Table 2

ChE Activity (Heat/Sarin-3months -experiment)
mean value (μ moles/min/mg protein)

Regions	name of the group							
	CONTROL		HEAT		SARIN		HEAT/SARIN	
	mean	SE	mean	SE	mean	SE	mean	SE
Cortex	1.83	0.42	1.73	0.37	1.64	0.36	2.03	0.5
Brainstem	1.52	0.08	1.84 *	0.1	1.82	0.12	1.82 *	0.03
Midbrain	1.79	0.07	1.85	0.16	1.72	0.37	1.94	0.11
Cerebellum	0.51	0.03	0.63 *	0.02	0.61	0.03	0.67 *	0.01
Plasma	0.03	0.005	0.04	0.01	0.08	0.01	0.04	0.01

ChE Activity (Heat/Sarin-3months-experiment)
(% of control)

Regions	name of the group							
	CONTROL		HEAT		SARIN		HEAT/SARIN	
	% of control	SE	% of control	SE	% of control	SE	% of control	SE
Cortex	100	22.65	94.5	20.22	89.2	19.47	110.5	27.32
Brainstem	100	5.5	121.4 *	6.72	120.2	7.65	120.4 *	2.14
Midbrain	100	4.01	103.6	9.01	96.1	20.94	108.3	6.32
Cerebellum	100	6.07	122.3 *	3.88	118.8	5.81	130.3 *	1.78
Plasma	100	9.84	139.5	24.32	268.6	43.31	134.6	34.91

Table 3

ChE Activity (Heat/Sarin-6months -experiment)
mean value (μ moles/min/mg protein)

Regions	name of the group							
	CONTROL		HEAT		SARIN		HEAT/SARIN	
	mean	SE	mean	SE	mean	SE	mean	SE
Cortex	1.57	0.32	1.07	0.18	1.19	0.14	1.56	0.28
Brainstem	1.45	0.04	1.66 *	0.04	1.49	0.1	1.61	0.06
Midbrain	1.21	0.18	1.78 *	0.13	1.75 *	0.11	1.8 *	0.04
Cerebellum	0.55	0.04	0.71 *	0.01	0.6	0.03	0.64	0.05
Plasma	0.08	0.01	0.1	0.01	0.13	0.03	0.16 *	0.03

ChE Activity (Heat/Sarin-6months-experiment)
(% of control)

Regions	name of the group							
	CONTROL		HEAT		SARIN		HEAT/SARIN	
	% of control	SE	% of control	SE	% of control	SE	% of control	SE
Cortex	100	20.27	67.8	11.51	75.7	9.09	99.3	17.84
Brainstem	100	3.02	114.6 *	2.69	102.8	7.05	111.2	4.36
Midbrain	100	14.58	147.4 *	10.77	144.9 *	9.35	148.8 *	3.43
Cerebellum	100	6.66	129.6 *	1.39	109.1	6.15	117.8	8.92
Plasma	100	19.36	132.6	14.71	167.9	34.75	217.1 *	35.93

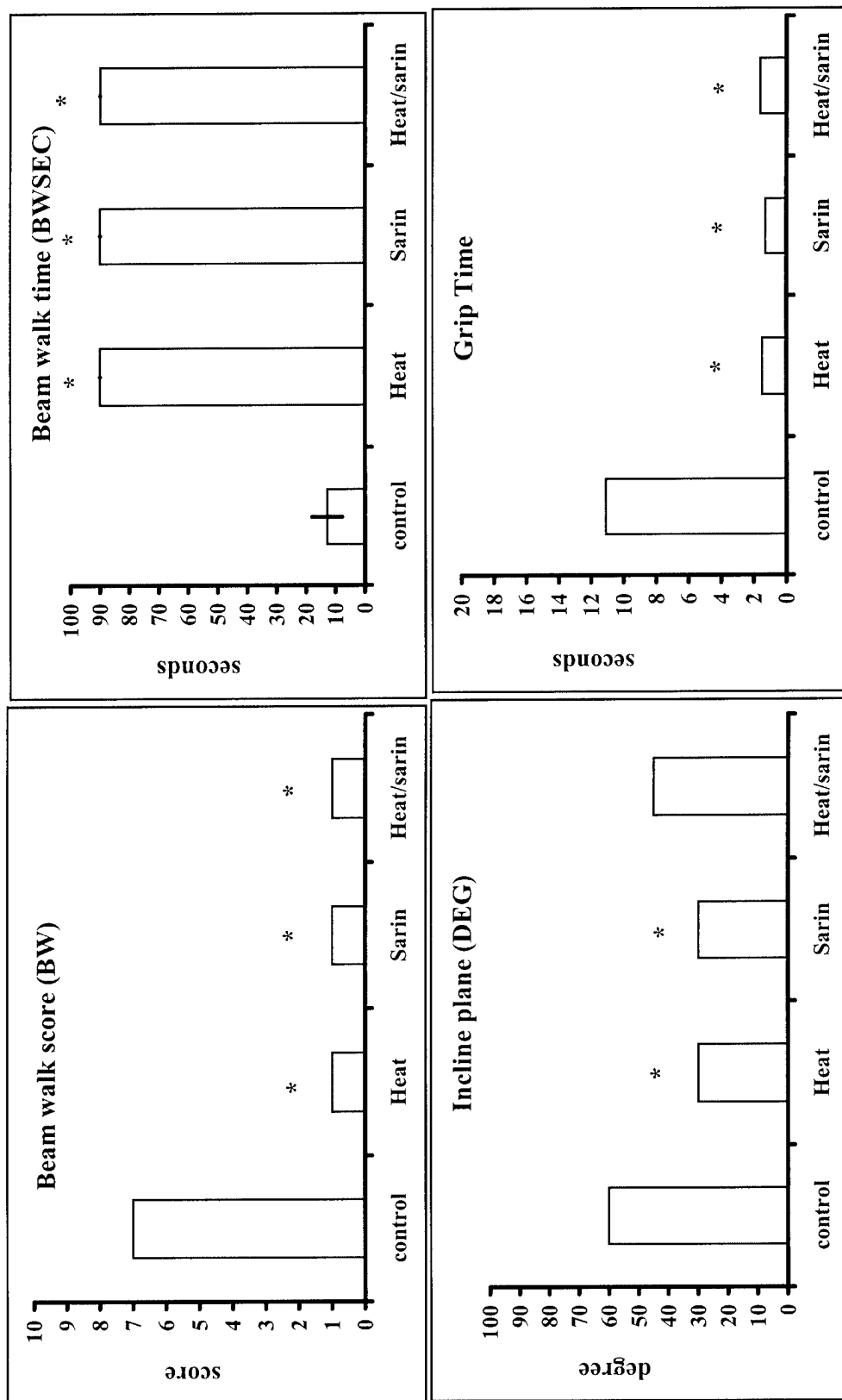


Figure 1 A : Behavioral studies (3 months)

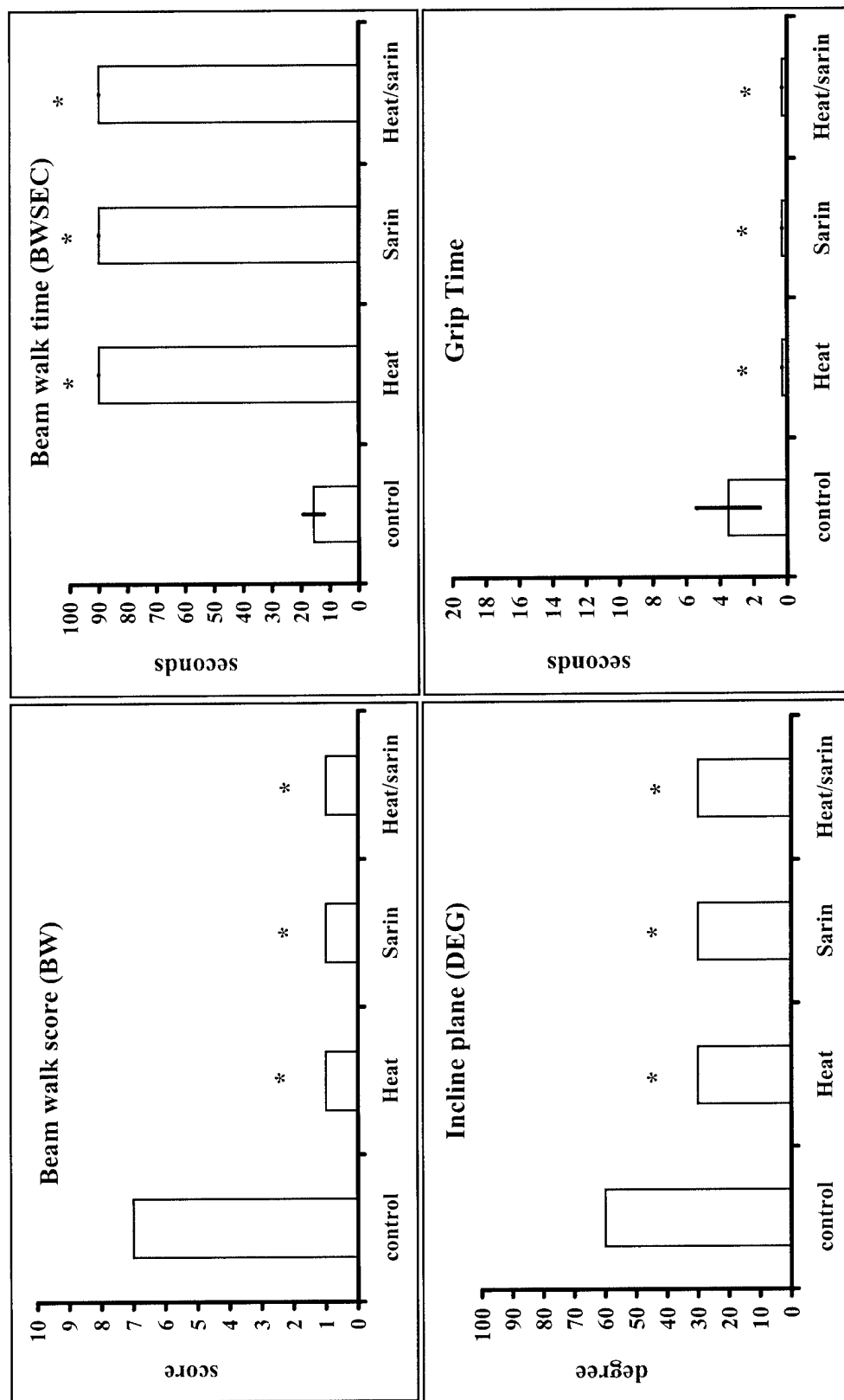


Figure 1 B : Behavioral studies (6 months)

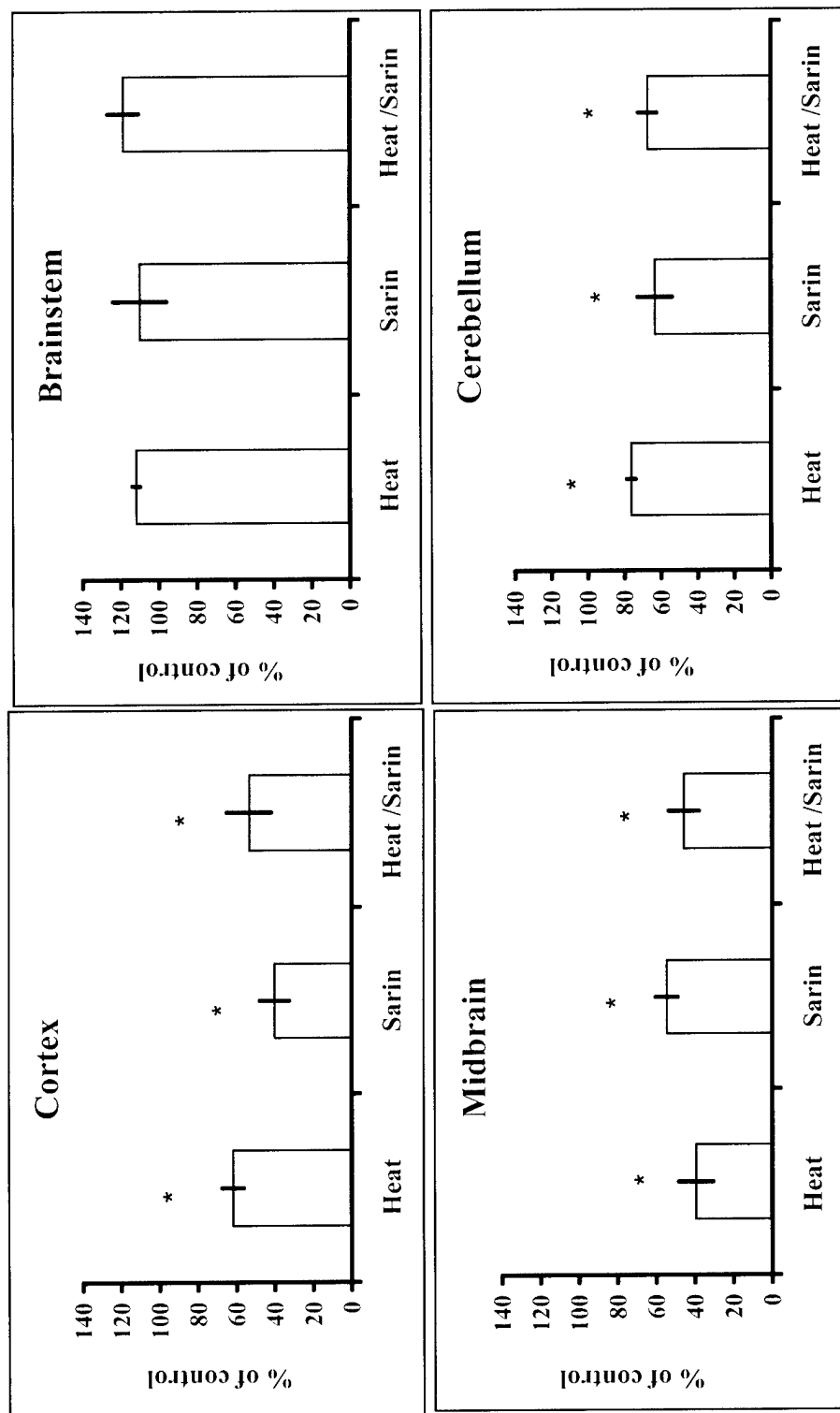


Figure 2 A : Brain regional AChE activity (Heat/sarin-24hr)

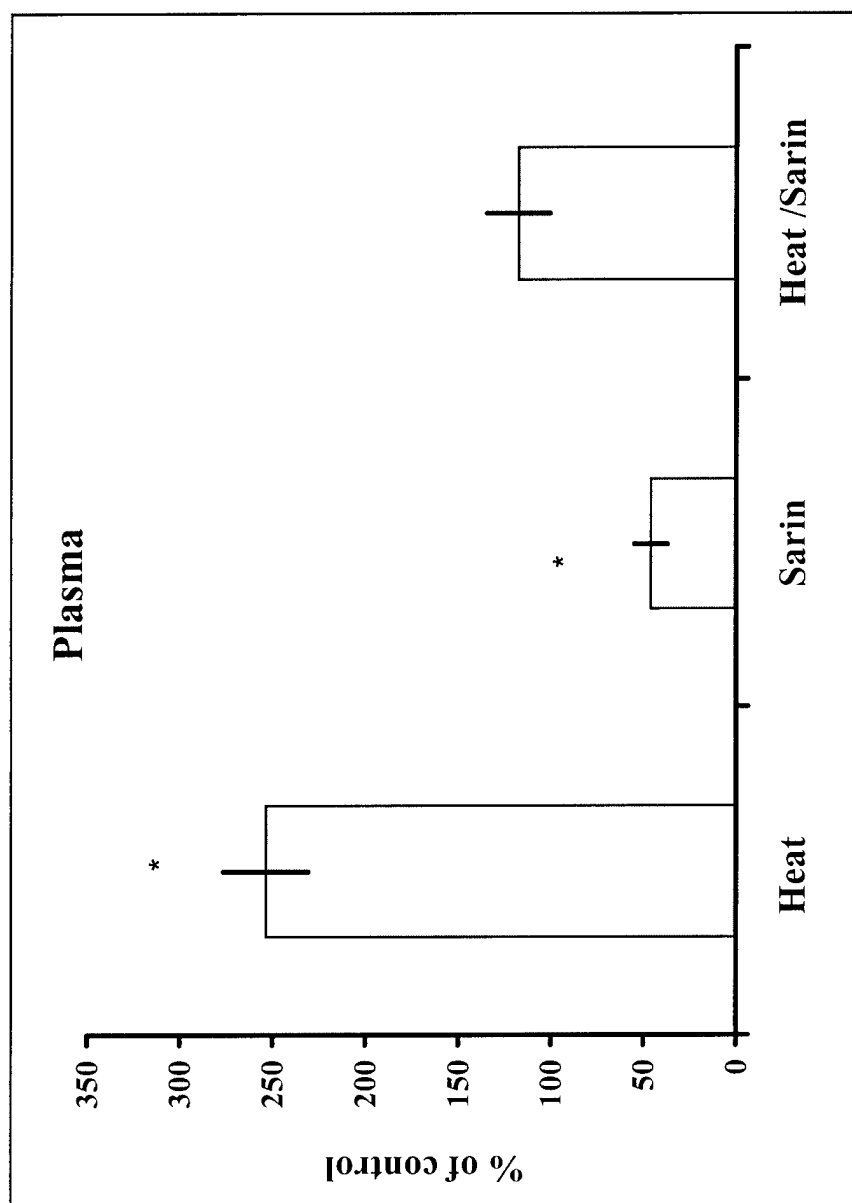


Figure 2 B : Plasma BChE activity (Heat/sarin-24hr)

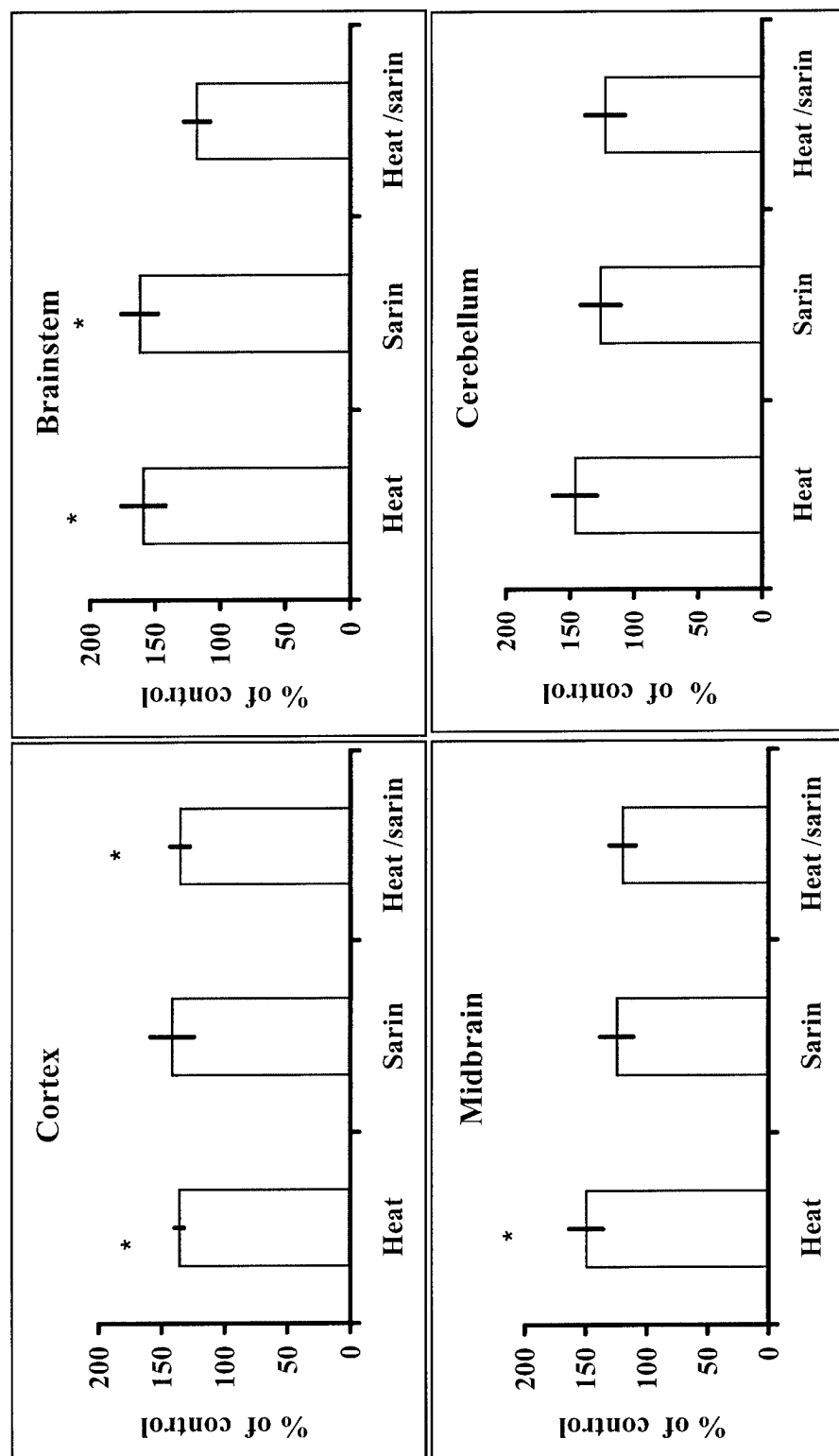


Figure 2 C : BBB permeability changes (Heat/sarin -24hr)

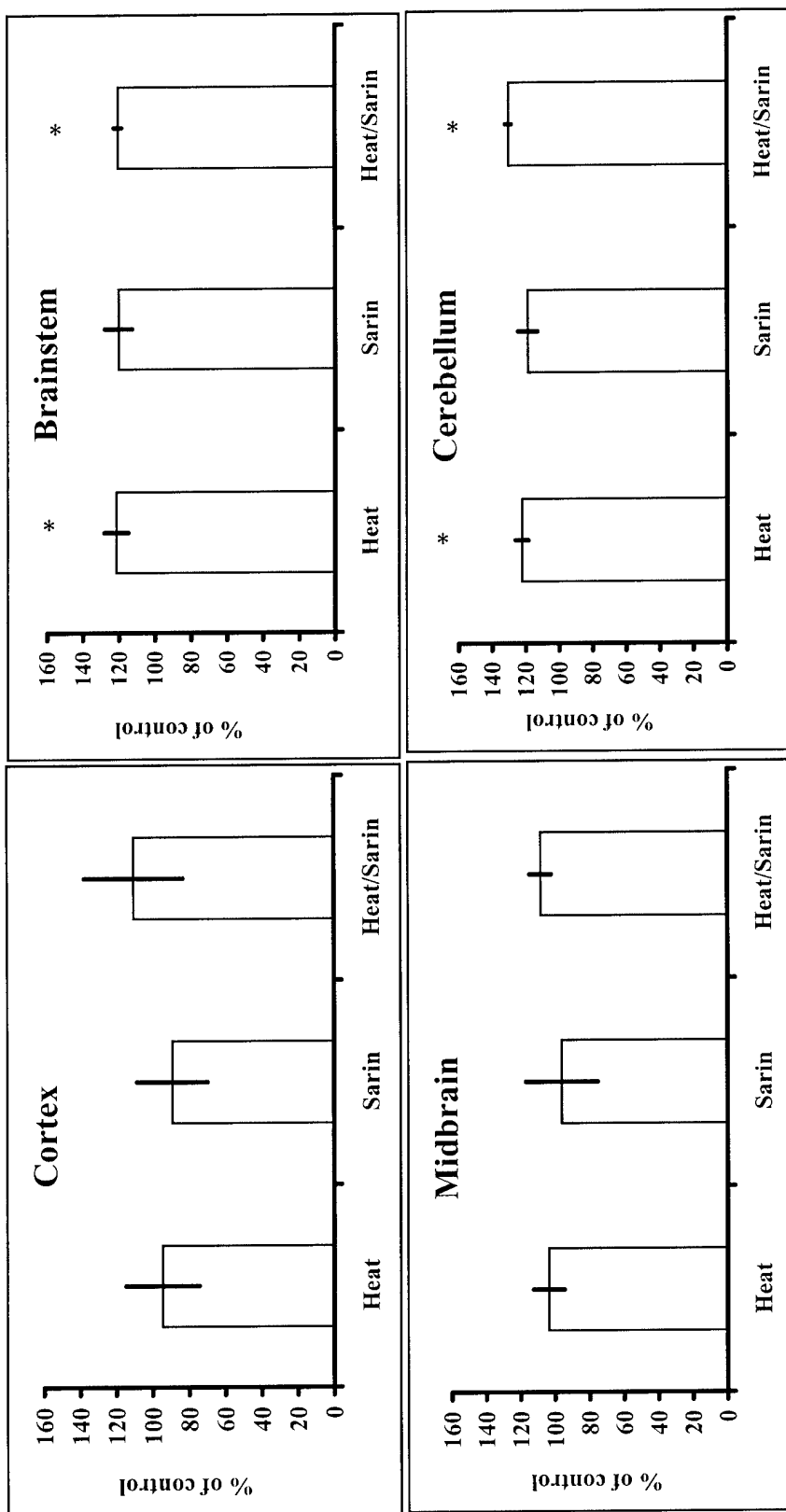


Figure 3 A : Brain regional AChE activity (Heat/sarin-3months)

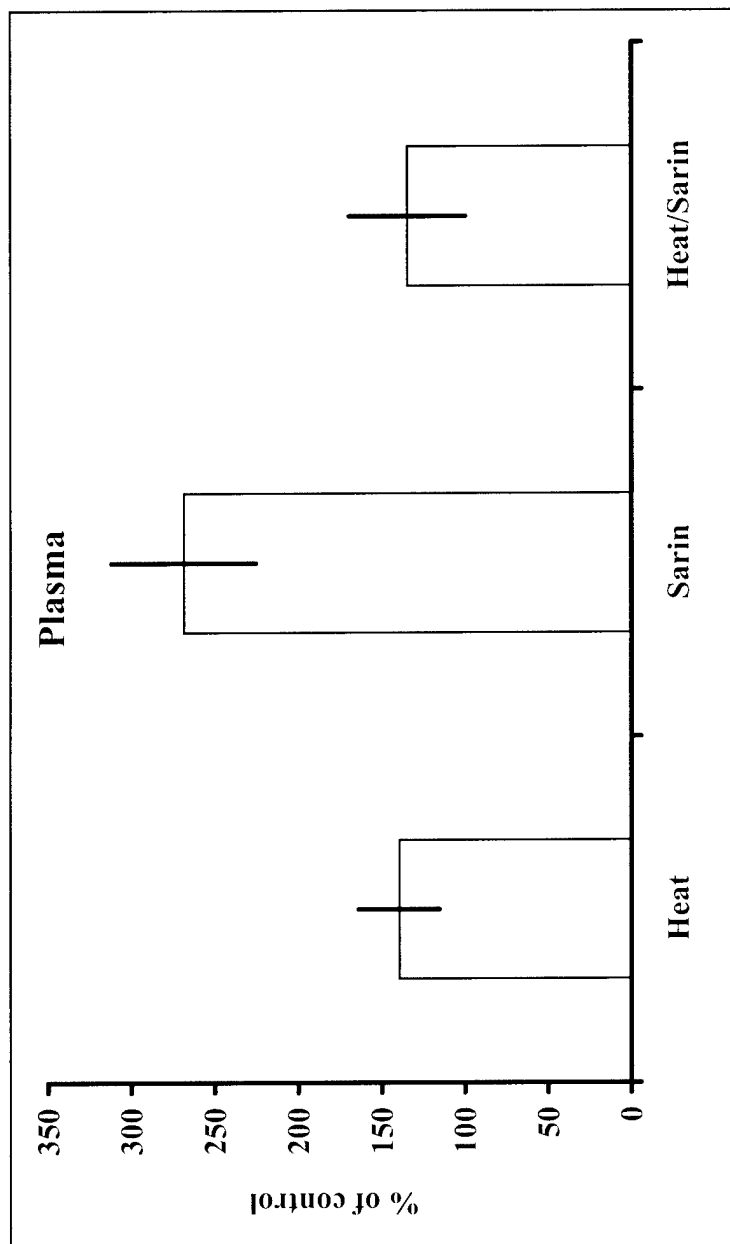


Figure 3 B : Plasma BChE activity (Heat/sarin-3months)

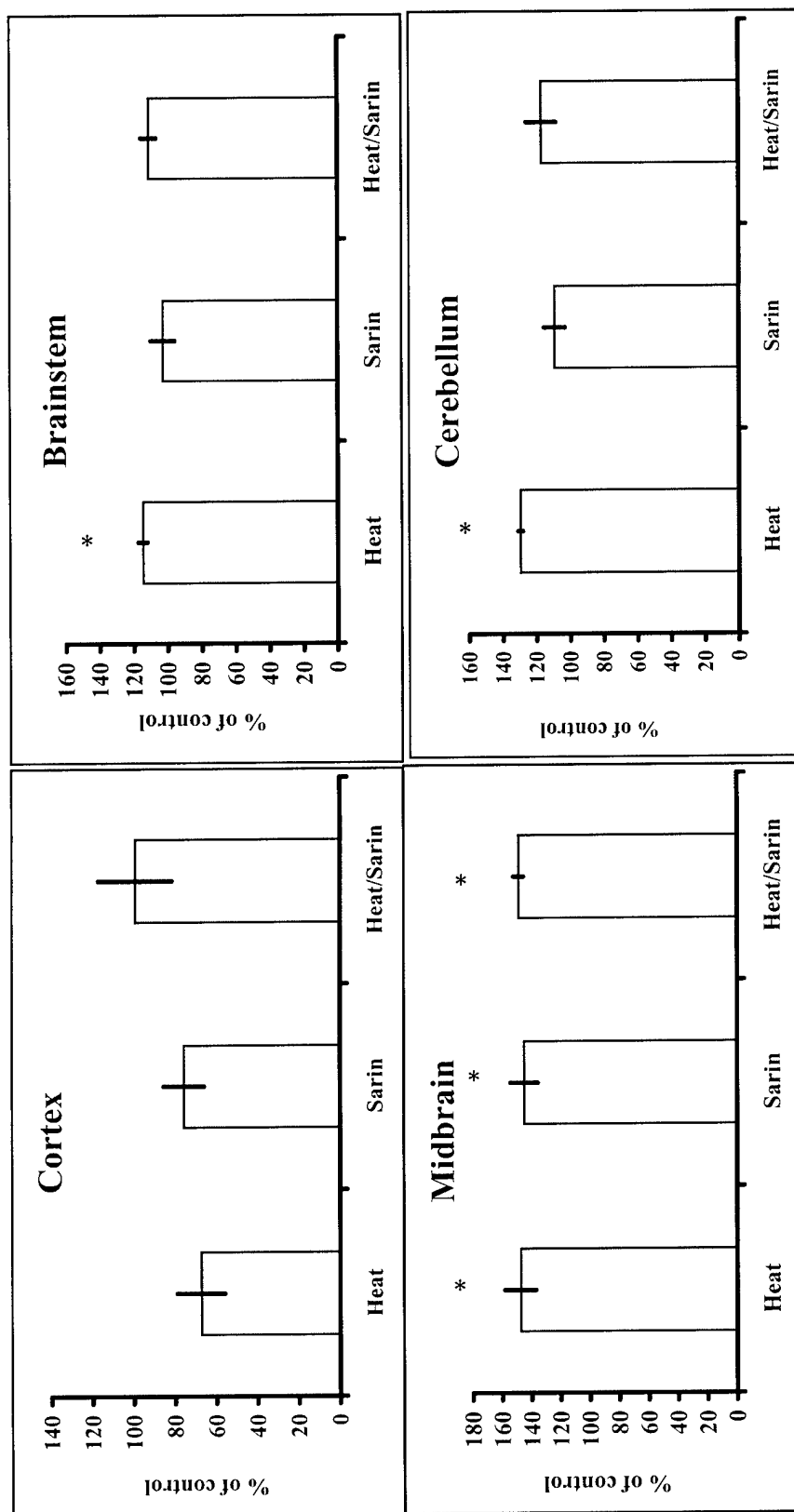


Figure 4 A : Brain regional AChE activity (Heat/sarin-6 months)

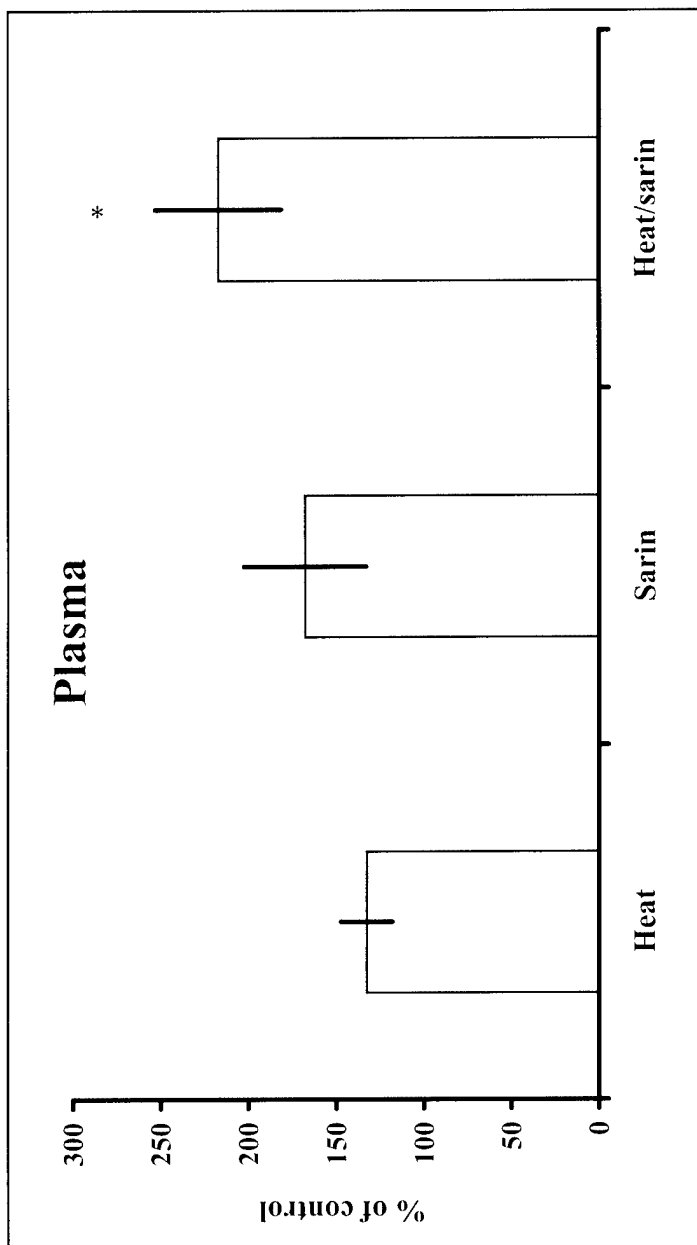


Figure 4 B : Plasma BChE activity (Heat/sarin-6 months)

**Specific Aim 4. Effect of prophylactic treatment with pyridostigmine bromide (PB)
on sub-clinical sarin exposure.**

Rationale: PB was given to PGW veterans prophylactically to protect acetylcholinesterase from the nerve agent poisoning. PB acts by reversibly inhibiting 30-40% of acetylcholinesterase in the peripheral nervous system, thus protecting the enzyme from irreversible inhibition by nerve agents (Blick *et al.*, 1991). However, there has been concern that PB may have played some role in the health problems experienced by the PGW veterans. The studies in this specific aim were carried out to evaluate the effects of PB on sarin-induced neurotoxicity.

The effects of PB and sarin alone, and in combination on sensorimotor behavior and the central cholinergic system of rats were studied. For these studies, the rats were treated with 15 daily oral dose of 1.3 mg/kg PB followed by a single i.m. dose of 0.5, 0.75, 0.9 or 1xLD₅₀ of sarin. Other group of rats was treated with PB or sarin alone. The animals were evaluated for postural reflexes, limb placing, orienting to vibrissae touch, incline plane performance, beam-walk time and forepaw grip time 7 and 15 days following treatment with sarin. Treatment with either PB or sarin alone resulted in significant sensorimotor impairments. Co-exposure to sarin and PB resulted in significant sensorimotor deficits that continued to worsen with time. At higher doses of sarin, co-exposure to PB resulted in exacerbated effect on sensorimotor functions. Cortical acetylcholinesterase (AChE) activity remained inhibited in the animals treated with each dose of sarin alone and in combination with PB. Cortex and brainstem muscarinic acetylcholine receptor (m2 mAChR) ligand binding showed significant increases (~120-130% of control) following co-exposure to PB and sarin at higher doses. Acute exposure to either 0.1x or 1x LD₅₀ sarin (single i.m. injection) for 3 hrs with or without 15 days of oral pretreatment with PB (1.3 mg/kg/day) showed significant

inhibition in plasma BChE activity following treatment with 1xLD50 sarin (~30% of control). Pretreatment with PB afforded slight protection. AChE activity in brain regions exhibited significant inhibition following treatment with 1xLD50 sarin and pretreatment with PB afforded a slight protection. However, the AChE activities in the brain regions of the animals treated with 1xLD50 sarin that were pretreated with PB, remained significantly inhibited as compared to the controls (Abou-Donia et al, 2001, published manuscript presented in appendix 1).

Oxidative stress has been recognized as a common denominator in a variety of neurodegenerative diseases. We explored the possibility of the generation of oxidative stress following exposure to sarin and PB, alone and in combination. Urinary levels of 3-nitrotyrosine and 8-hydroxy-2'-deoxyguanosine, as markers of oxidative stress, were determined by high performance liquid chromatography. Treatment of the rats with a combination of a single oral dose of PB (13 mg/kg, gavage) and a single i.m. dose of sarin (80µg/kg) resulted in a significantly increased levels of urinary levels of 3-nitrotyrosine and 8-hydroxy-2'-deoxyguanosine at 48 hrs after the treatment and stayed elevated upto 96 hrs following the treatment. Treatment with either PB or sarin alone did not cause an increased excretion of these biomarkers of oxidative stress in the urine (Abu-Qare and Abou-Donia, 2001, published manuscript presented in appendix 1).

Experimental design for subclinical exposure to sarin, alone and in combination with PB for 24 hrs, 3 months and 6 months time points:

Materials and Methods: Male Sprague-Dawley rats (225-250g) were obtained from Zivic-Miller Laboratories, Allison Park, PA. The animals were housed in Duke University Medical Center vivarium at 21-23 °C and on a 12-h dark-light cycle. The

animals were allowed food and water *ad libitum*. All the treatments and procedures on the animals were carried out strictly according to the recommended guidelines by the Duke University Institutional Animal Care and Use Committee and U.S. Department of Defense.

Treatment of Animals and Tissue Retrieval

Animals were randomly divided into following groups of 5 animals each for each time point of the study.

Control: The rats treated daily with oral water for 15 days and then treated with single i.m. injection of saline.

PB alone: The rats were treated with 1.3 mg/kg PB (in water, oral by gavage), daily for 15 days.

Sarin alone: The rats were treated with single i.m. injection of 0.5xLD₅₀ sarin.

Co-exposure to PB and sarin: The rats were treated with 1.3 mg/kg PB (in water, oral by gavage), daily for 15 days. On 15th day following treatment with PB, the rats were treated with single i.m. injection of 0.5xLD₅₀ sarin.

At 24 hrs, 3 and 6 month following treatment with sarin, the rats were anesthetized with ketamine/xylazine (100mg/kg ketamine, 15mg/kg xylazine) and blood was drawn in heparinized syringe. Brains were removed and washed thoroughly with ice cold normal saline to remove blood. Brain regions, cortex, midbrain, cerebellum and brainstem were dissected on ice and snap frozen in liquid nitrogen. Plasma was separated and frozen at -80 °C for enzyme studies.

Behavioral Testing Battery:

The behavioral tests employed in these studies evaluate sensorimotor reflexes, motor strength, and coordination. All behavioral testing was performed by an observer blind to the animal's treatment status and was carried out in a sound-proof room with subdued lighting (less than 10.76 lumens/m², ambient light) between 7 and 11:30 AM. Behavioral testing was carried out at 3 and 6 months following single i.m. injection of 0.5xLD₅₀ sarin as described in specific aim 2.

Statistical Analyses

Comparisons across treatment groups for postural reflexes, limb placing, and vibrissae touch orientation were analyzed with non-parametric analysis of variance (Kruskal-Wallis test). Data for the remaining behavioral tests were compared among groups by one-way or two-way repeated measures ANOVA as appropriate. If a significant difference was found, Fisher's LSD tests were applied to permit post-hoc, pair-wise comparisons. A two-tailed P value of <0.05 was considered statistically significant.

Cholinesterase determination:

Activities of AChE in brain regions and BChE in plasma were determined in a Molecular Devices UV Max Kinetic Microplate Reader, as described in specific aim 2. The enzyme activities are expressed as μ mole substrate hydrolyzed/min/mg protein.

Statistical Analysis:

The results were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test. P value <0.05 was considered significant.

Results and conclusions: The data are presented in appendix 4. Neurobehavioral evaluations for sensorimotor performance are presented in Fig 1 A& B for 3 and 6

months, respectively. Beam-walk score showed no significant impairments at 90 days following either treatment PB or sarin alone. At 6 months, there was significant impairment in the animals treated with PB alone. For beam-walk time, incline plane and fore-paw grip time, there was significant effects of treatment groups at both the time points. However, performance over time was variable. However, there was no dramatic additive effect when the rats were exposed to sarin and PB in combination.

Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) are presented in Fig (2–4) for 24 hrs, 3 months and 6 months, respectively. Treatment with sarin alone did not cause any significant change in brain regional or plasma cholinesterase activity. Brain regional AChE and plasma BChE activity from 3 month group of rats are presented in Fig 3. There was a significant increase in cortex AChE activity (~159% of control) following treatment with sarin alone. Figure 4 demonstrates the brain regional AChE and plasma BChE activity 6 months after sarin treatment. There was a significant increase in midbrain AChE activity (~119% of control) in the animals treated with PB alone and an increase in cerebellum AChE activity in the animals treated with sarin alone. Treatment with a combination of PB and sarin did not result in any significant change in brain regional AChE activity at 6 month time point. These results suggest that exposure to a combination of PB and sarin does not cause long-term changes in the cholinesterase activity in brain regions.

Figure Legends:

Figure 1: Sensorimotor performance following a single i.m. treatment with 0.5x LD50 sarin, alone or in combination with pyridostigmine bromide (PB). **A:** After 3 month of sarin treatment; **B:** After 6 month of sarin treatment. The rats were treated with 1.3 mg/kg PB (in water, oral by gavage), daily for 15 days. The control and sarin group rats were treated with water (oral by gavage). On day 15, following the last treatment with PB, the rats were treated with a single i.m. injection of 0.5xLD50 sarin. The rats from control and PB alone group were treated with i.m. injection of normal saline. The rats were maintained for a further period of 3 and 6 months. The animals were evaluated for beam-walk score, beam-walk time, incline plane and grip response. The data are presented as mean \pm SE, n=5. *Indicates statistically significant.

Figure 2: Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) activity 24 hrs after single i.m. treatment with 0.5x LD50 sarin, alone and in combination with PB. **A:** AChE activity in different brain regions; **B:** BChE activity in plasma. The rats were treated with 1.3 mg/kg PB (in water, oral by gavage), daily for 15 days. The control and sarin group rats were treated with water (oral by gavage). On day 15, following the last treatment with PB, the rats were treated with a single i.m. injection of 0.5xLD50 sarin. The rats from control and PB alone group were treated with i.m. injection of normal saline. The rats were sacrificed 24 hrs after the treatment with sarin. Enzyme activity was determined as described in Materials and Methods. The control activity was 0.79 ± 0.11 cortex; 1.39 ± 0.06 brainstem; 1.39 ± 0.06 midbrain; 0.73 ± 0.04 cerebellum and 0.03 ± 0.001 , plasma expressed as μ moles

substrate hydrolyzed/min/mg protein. The data are presented as mean + SE (% of control), n = 5. * Indicates statistically significant.

Figure 3: Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) 3 months after a single i.m. treatment with 0.5x LD50 sarin. **A:** AChE activity in different brain regions; **B:** BChE activity in plasma. The rats were treated with 1.3 mg/kg PB (in water, oral by gavage), daily for 15 days. The control and sarin group rats were treated with water (oral by gavage). On day 15, following the last treatment with PB, the rats were treated with a single i.m. injection of 0.5xLD50 sarin. The rats from control and PB alone group were treated with i.m. injection of normal saline. The rats were maintained for a further period of 3 months. Enzyme activity was determined as described in Materials and Methods. The control activity was 0.88 ± 0.06 cortex; 2.01 ± 0.16 brainstem; 1.38 ± 0.08 midbrain; 0.48 ± 0.02 cerebellum and 0.039 ± 0.001 , plasma expressed as μ moles substrate hydrolyzed/min/mg protein. The data are presented as mean + SE (% of control), n = 5. * Indicates statistically significant.

Figure 4: Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) 6 months after a single i.m. treatment with 0.5x LD50 sarin. **A:** AChE activity in different brain regions; **B:** BChE activity in plasma. The rats were treated with 1.3 mg/kg PB (in water, oral by gavage), daily for 15 days. The control and sarin group rats were treated with water (oral by gavage). On day 15, following the last treatment with PB, the rats were treated with a single i.m. injection of 0.5xLD50 sarin. The rats from control and PB alone group were treated with i.m. injection of normal saline. The rats were maintained for a further period of 6 months. Enzyme activity

was determined as described in Materials and Methods. The control activity was 1.27 ± 0.28 cortex; 1.48 ± 0.09 brainstem; 1.20 ± 0.05 midbrain; 0.5 ± 0.02 cerebellum and 0.06 ± 0.01 , plasma expressed as μ moles substrate hydrolyzed/min/mg protein. The data are presented as mean + SE (% of control), n = 5. * Indicates statistically significant.

Table 1

ChE Activity (PB/sarin-24hr)
mean value (μ moles/min/mg protein)

Regions	name of the group							
	control		PB		SARIN		PB/SARIN	
	mean	SE	mean	SE	mean	SE	mean	SE
Cortex	0.79	0.11	0.98	0.17	0.6	0.13	0.89	0.08
Brainstem	1.39	0.21	1.79	0.05	1.88	0.1	1.54	0.11
Midbrain	1.39	0.06	1.36	0.05	1.56	0.08	1.41	0.16
Cerebellum	0.73	0.04	0.73	0.03	0.72	0.07	0.63	0.02
Plasma	0.03	0.001	0.02	0.001	0.03	0.001	0.03	0.001

ChE Activity (PB/sarin-24hr)
(% of control)

Regions	name of the group							
	control		PB		SARIN		PB/SARIN	
	% of control	SE	% of control	SE	% of control	SE	% of control	SE
Cortex	100	13.4	123.9	21.9	76.7	15.9	113	9.7
Brainstem	100	14.9	128.4	3.4	135.5	7.4	110.9	8
Midbrain	100	4	98	3.6	112.7	6.1	101.8	11.5
Cerebellum	100	5.9	98.9	3.4	98.7	10.1	86.4	2.6
Plasma	100	16.5	85.2	7.9	113.3	14.4	94.5	15.6

Table 2

ChE Activity (PB/sarin-3months)
mean value (μ moles/min/mg protein)

Regions	name of the group							
	control		PB		SARIN		PB/SARIN	
	mean	SE	mean	SE	mean	SE	mean	SE
Cortex	0.88	0.065	1.46	0.4	1.4*	0.24	1.27	0.34
Brainstem	2.01	0.16	1.63	0.1	1.61	0.18	1.81	0.08
Midbrain	1.38	0.08	1.68	0.1	1.4	0.1	1.56	0.12
Cerebellum	0.48	0.02	0.42	0.02	0.48	0.3	0.45	0.04
Plasma	0.039	0.001	0.036	0.001	0.038	0.01	0.029	0.01

ChE Activity (PB/sarin-3months)
(% of control)

Regions	name of the group							
	control		PB		SARIN		PB/SARIN	
	% of control	SE	% of control	SE	% of control	SE	% of control	SE
Cortex	100	7.4	166.1	51	159.9 *	27.4	144.1	38.7
Brainstem	100	8	81.2	5.2	79.9	8.7	90.2	4.1
Midbrain	100	6	122.6	7.4	102.6	7.3	113.7	9.1
Cerebellum	100	3.9	87.8	4.5	100	6.5	93.6	8.7
Plasma	100	3.4	91.6	5	98.2	20.1	73.7	19.3

Table 3

ChE Activity (PB/sarin-6months)
mean value (μ moles/min/mg protein)

Regions	name of the group							
	control		PB		SARIN		PB/SARIN	
	mean	SE	mean	SE	mean	SE	mean	SE
Cortex	1.27	0.28	0.69	0.09	1.33	0.43	0.73	0.17
Brainstem	1.48	0.09	1.42	0.14	1.48	0.07	1.48	0.1
Midbrain	1.2	0.05	1.41*	0.07	1.59	0.27	1.37	0.13
Cerebellum	0.5	0.02	0.45	0.06	0.62 *	0.03	0.53	0.05
Plasma	0.06	0.01	0.08	0.01	0.07	0.01	0.04	0.01

ChE Activity (PB/sarin-6months)
(% of control)

Regions	name of the group							
	control		PB		SARIN		PB/SARIN	
	% of control	SE	% of control	SE	% of control	SE	% of control	SE
Cortex	100	22.4	54.2	7	105.2	34.6	57.8	13
Brainstem	100	6.3	96.1	9.3	99.6	4.9	100.3	6.8
Midbrain	100	4.3	119.1*	6	134.7	22.6	115.3	10.6
Cerebellum	100	4.3	91.3	12.7	124.8 *	6.5	106.2	10.7
Plasma	100	14.4	135.3	10.1	117.9	9.4	60	14.6

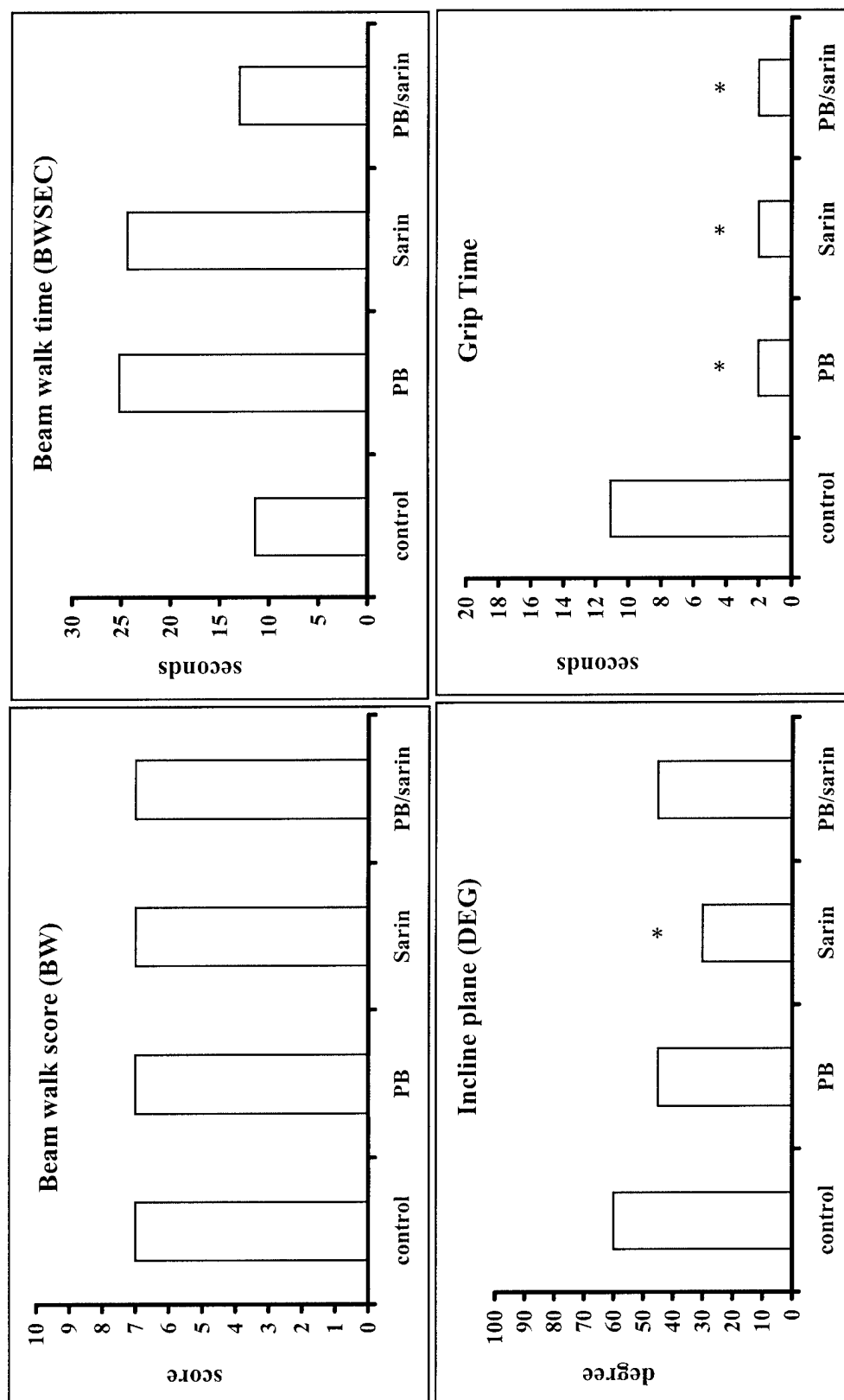


Figure 1 A : Behavioral studies (3 months)

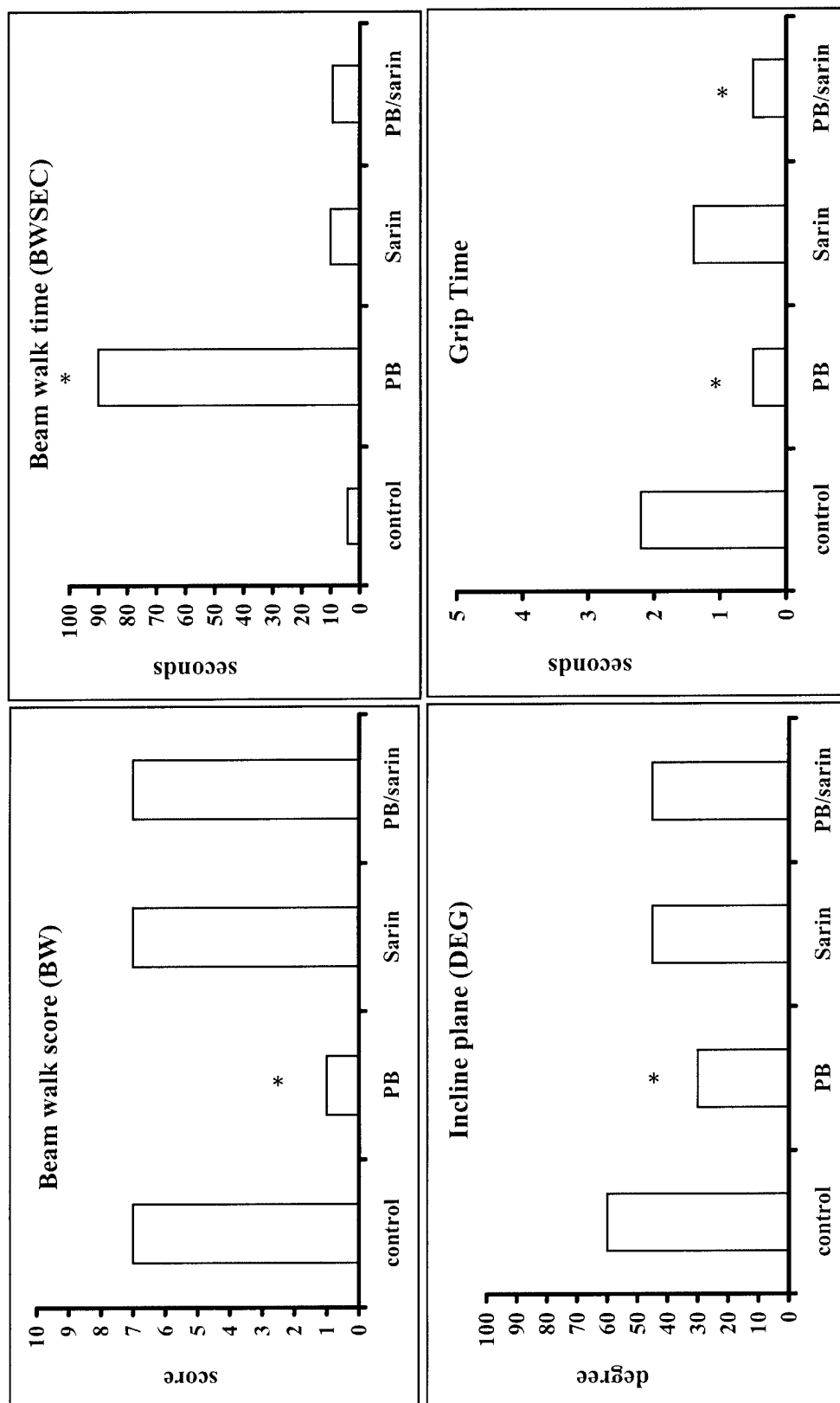


Figure 1 B : Behavioral studies (6 months)

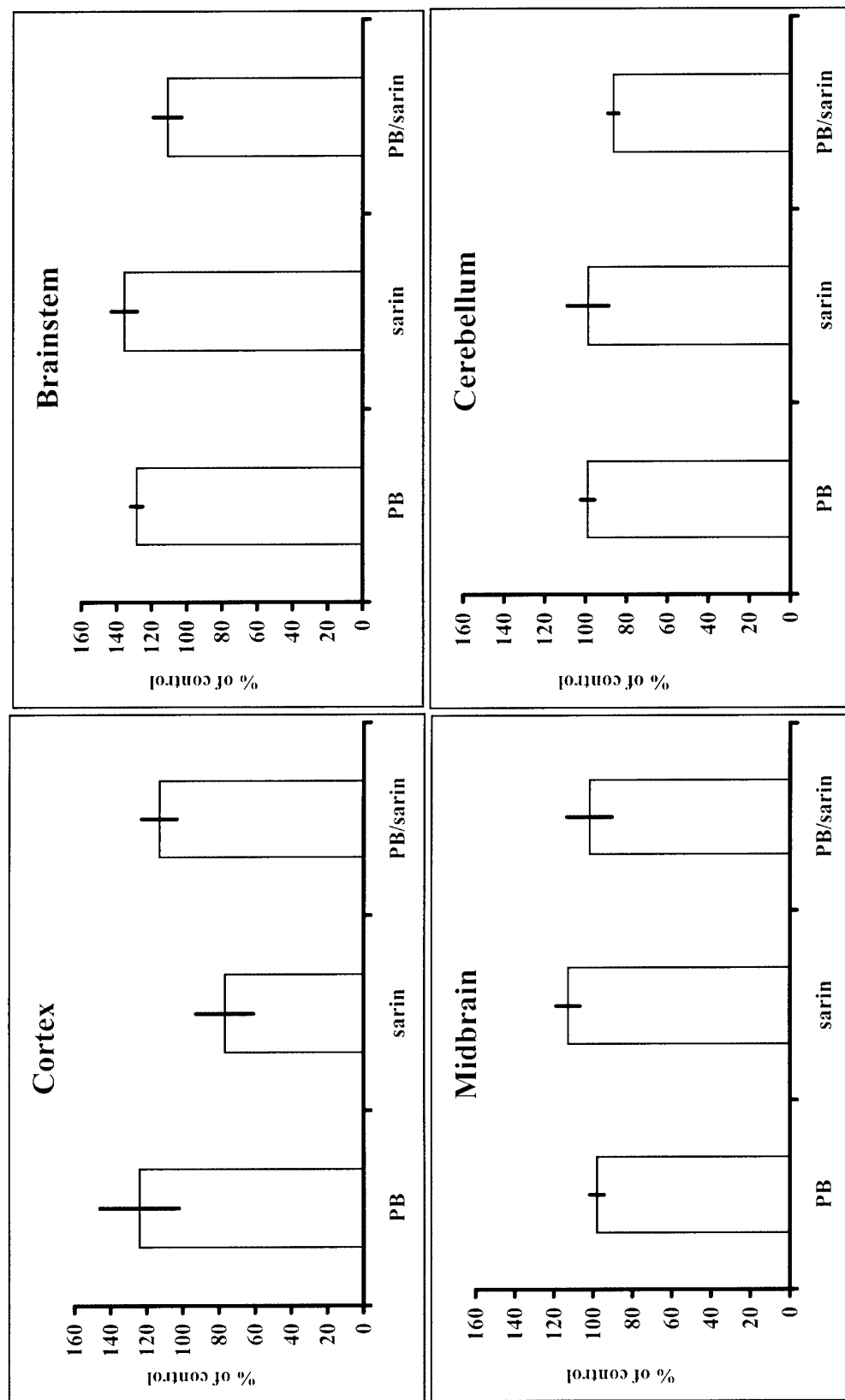


Figure 2 A : Brain regional AChE activity (PB/sarin-24 hr)

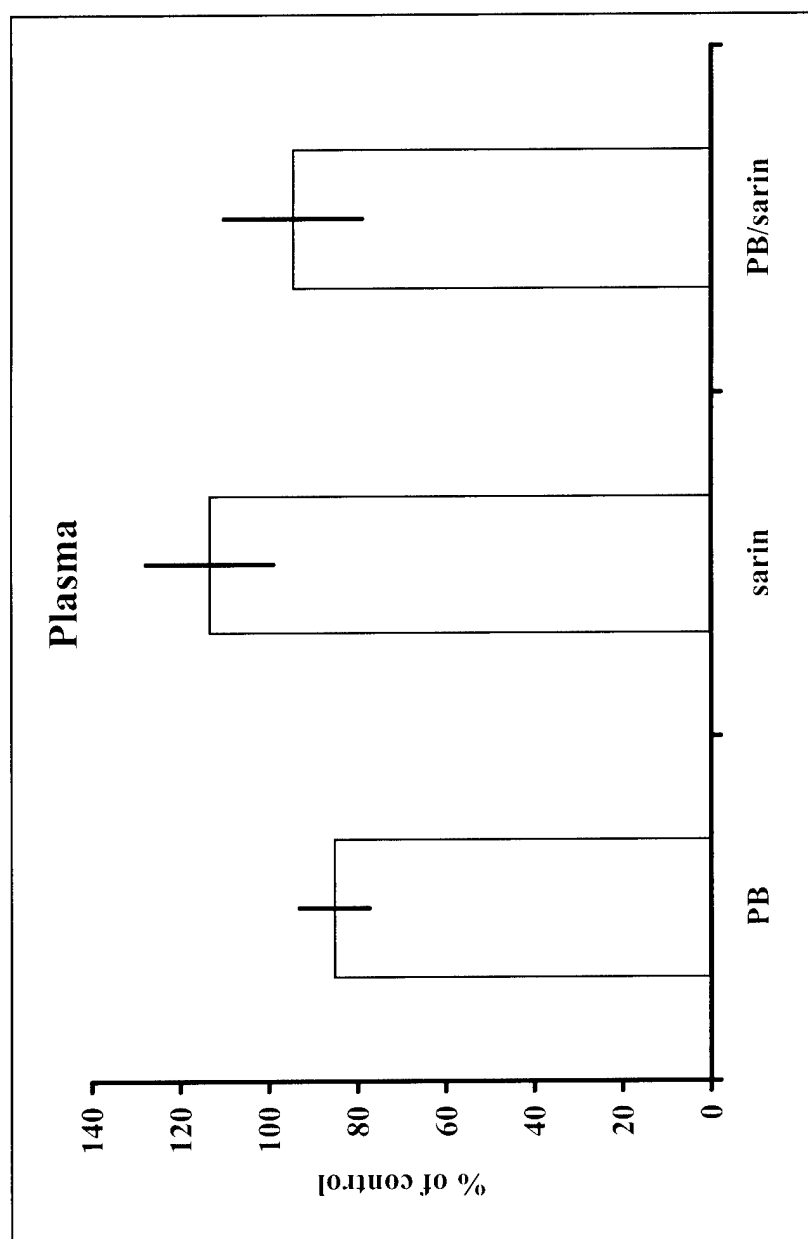


Figure 2 B : Plasma BChE activity (PB/sarin-24 hr)

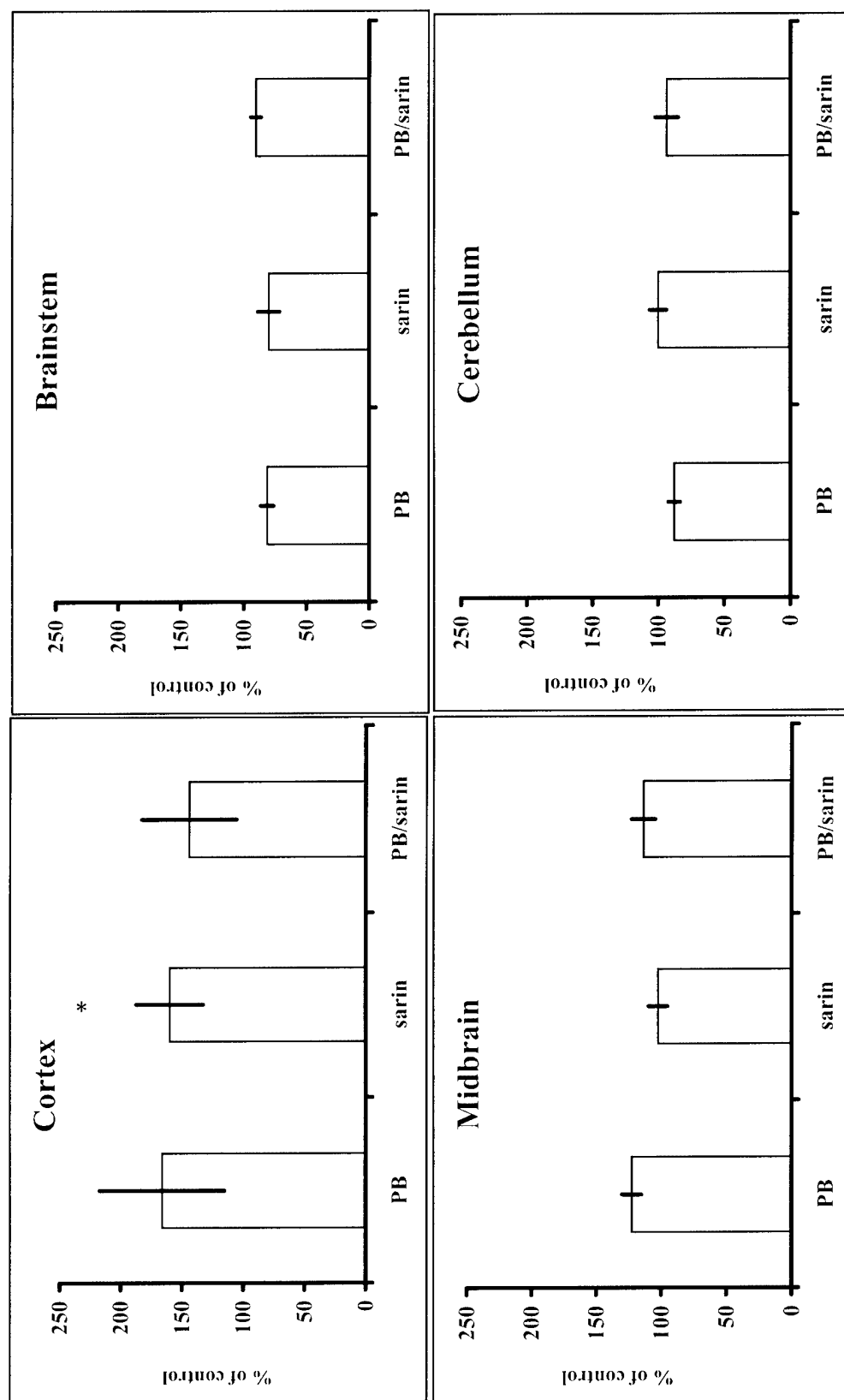


Figure 3 A : Brain regional AChE activity (PB/sarin - 3months)

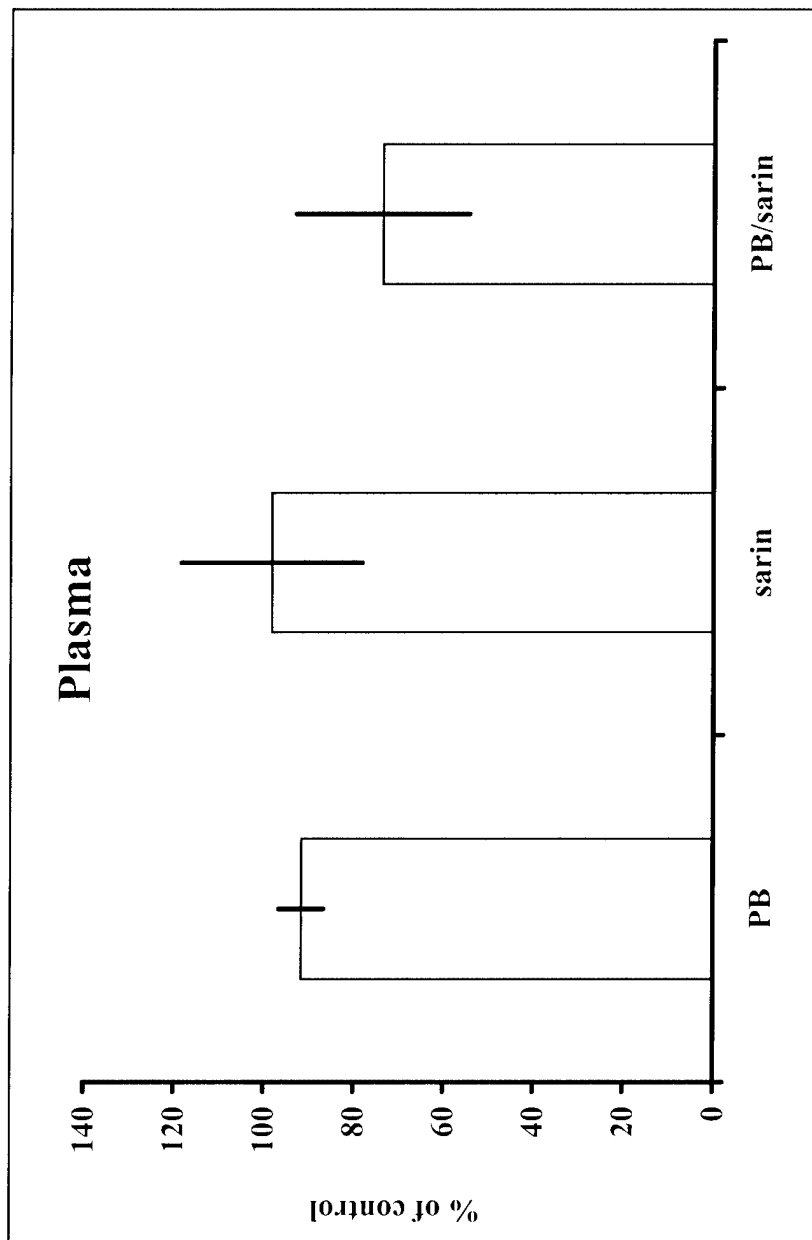


Figure 3 B : Plasma BChE activity (PB/sarin - 3months)

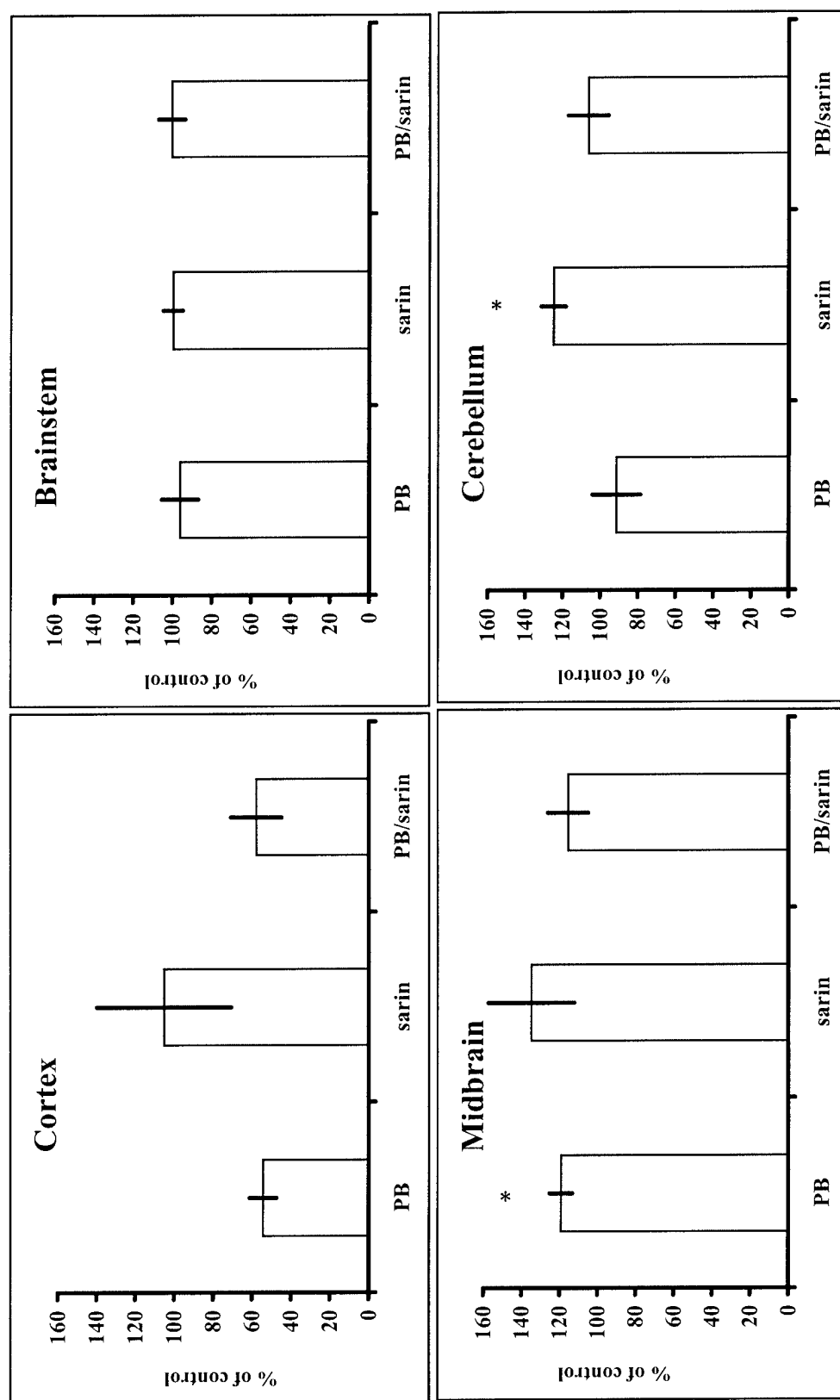


Figure 4 A : Brain regional AChE activity (PB/sarin-6 months)

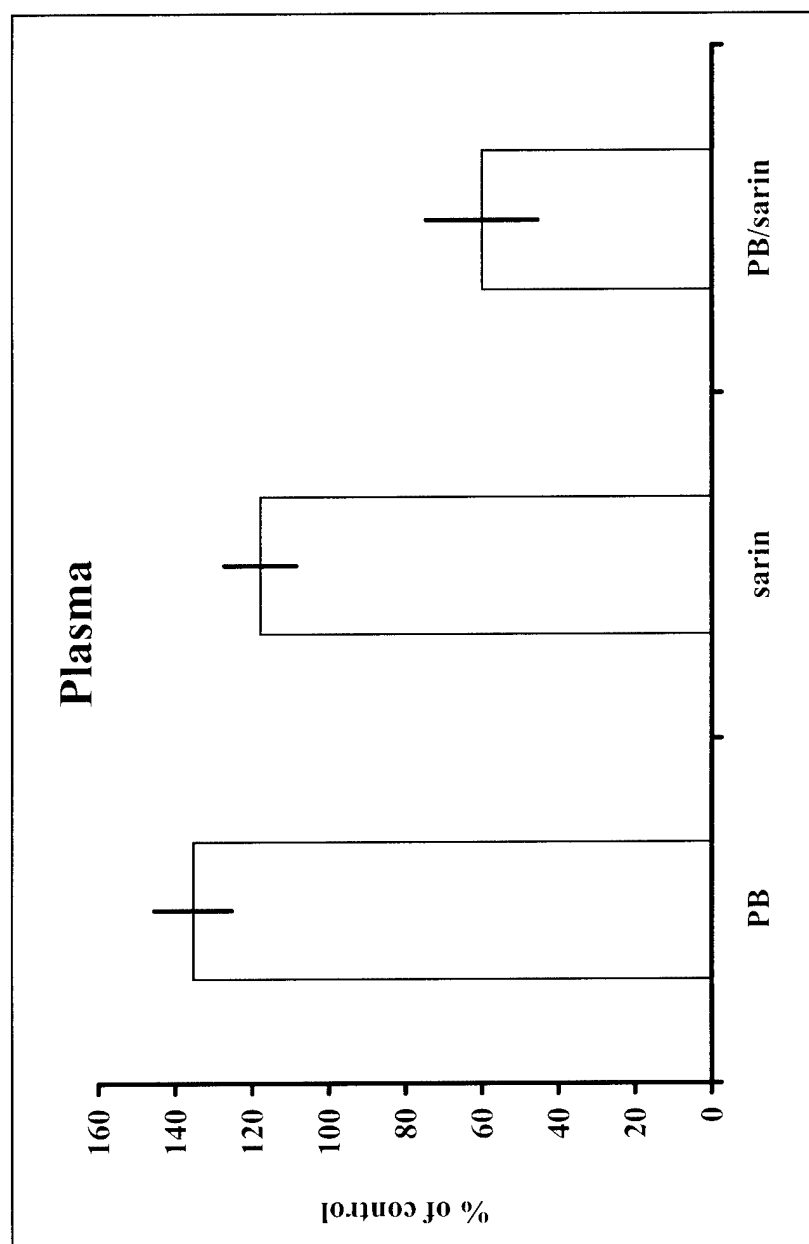


Figure 4 B : Plasma BChE activity (PB/sarin-6 months)

Specific Aim 5. Effect of treatment with PB after sub-clinical sarin exposure.

Rationale: PB was given to the participating military personnel prophylactically to protect against nerve agent attack. In some case however, it is also possible that PB was taken by the deployed personnel only after exposure to sarin might have occurred. These studies were carried out to evaluate the possible effects of PB following exposure to sarin.

Materials and Methods: Male Sprague-Dawley rats (225-250g) were obtained from Zivic-Miller Laboratories, Allison Park, PA. The animals were housed in Duke University Medical Center vivarium at 21-23 °C and on a 12-h dark-light cycle. The animals were allowed food and water *ad libitum*. All the treatments and procedures on the animals were carried out strictly according to the recommended guidelines by the Duke University Institutional Animal Care and Use Committee and U.S. Department of Defense.

Treatment of Animals and Tissue Retrieval

Animals were randomly divided into following groups of 5 animals each for each time point of the study.

Control: The rats treated with single i.m. injection of saline, followed by daily treatment with oral water for 15 days.

PB alone: The rats treated with single i.m. injection of saline, followed by treatment with 1.3 mg/kg PB (in water, oral by gavage), daily for 15 days.

Sarin alone: The rats were treated with single i.m. injection of 0.5xLD₅₀ sarin, followed by daily treatment with oral water by gavage for 15 days.

Co-exposure to PB and sarin: The rats were treated with single i.m. injection of 0.5xLD50 sarin, followed by daily treatment with 1.3 mg/kg PB (in water, oral by gavage), daily for 15 days.

At 24 hrs, 3 and 6 month following the treatment with the last dose of PB, the rats were anesthetized with ketamine/xylazine (100mg/kg ketamine, 15mg/kg xylazine) and blood was drawn in heparinized syringe. Brains were removed and washed thoroughly with ice cold normal saline to remove blood. Brain regions, cortex, midbrain, cerebellum and brainstem were dissected on ice and snap frozen in liquid nitrogen. Plasma was separated and frozen at -80 °C for enzyme studies.

Behavioral Testing Battery:

The behavioral tests employed in these studies evaluate sensorimotor reflexes, motor strength, and coordination. All behavioral testing was performed by an observer blind to the animal's treatment status and was carried out in a sound-proof room with subdued lighting (less than 10.76 lumens/m², ambient light) between 7 and 11:30 AM. Behavioral testing was carried out at 3 and 6 months following single i.m. injection of 0.5xLD50 sarin as described in specific aim 2.

Statistical Analyses

Comparisons across treatment groups for postural reflexes, limb placing, and vibrissae touch orientation were analyzed with non-parametric analysis of variance (Kruskal-Wallis test). Data for the remaining behavioral tests were compared among groups by one-way or two-way repeated measures ANOVA as appropriate. If a significant difference was found, Fisher's LSD tests were applied to permit post-hoc,

pair-wise comparisons. A two-tailed P value of <0.05 was considered statistically significant.

Cholinesterase determination:

Activities of AChE in brain regions and BChE in plasma were determined in a Molecular Devices UV Max Kinetic Microplate Reader, as described in specific aim 2. The enzyme activities are expressed as μ mole substrate hydrolyzed/min/mg protein.

Statistical Analysis:

The results were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test. P value <0.05 was considered significant.

Results and conclusions: The data are presented in appendix 5. Neurobehavioral evaluations for sensorimotor performance are presented in Fig 1 A& B for 3 and 6 months, respectively. At 3 months, beam-walk score showed a significant impairments following either treatment with sarin alone and PB in combination with sarin. At 6 months, there was significant impairment in the animals treated with PB alone or PB in combination with sarin. For beam-walk time, animals treated with PB or PB in combination with sarin performed poorly. Incline plane and fore-paw grip time, there was significant effects of treatment groups at both the time points. However, performance over time was variable. However, there was no dramatic additive effect following exposure to a combination of sarin and PB.

Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) are presented in Fig (2–4) for 24 hrs, 3 months and 6 months, respectively. Figure 2 represents the brain regional AChE and plasma BChE activity 24 hrs after the last dose of PB. Treatment with sarin alone caused a significant

increase in brainstem and midbrain AChE activity (~208 and 217% of control) whereas treatment with a combination of PB and sarin caused a significant increase (~223% of control) in brainstem AChE activity. Brain regional AChE and plasma BChE activity from 3 month group of rats are presented in Fig 3. There was a significant increase in brainstem and midbrain AChE activity (~108 and 117%% of control for brainstem and ~121 and 130% of control for midbrain)) following treatment with sarin and PB alone. Brainstem also exhibited a significant increase (~116% of control) in AChE activity following combined treatment with PB and sarin. Figure 4 demonstrates the brain regional AChE and plasma BChE activity 6 months after the treatment with the last dose of PB. There was a significant increase in brainstem AChE activity (~112 and 130% of control) in the animals treated with PB and sarin alone. Treatment with a combination of PB and sarin did not result in any significant change in brain regional AChE activity at 6 month time point. These results suggest that exposure to a combination of PB and sarin, when PB was given after exposure to sarin, does not cause long-term changes in the cholinesterase activity in brain regions.

Figure Legends:

Figure 1: Sensorimotor performance following a single i.m. treatment with 0.5x LD50 sarin, alone or in combination with pyridostigmine bromide (PB). The treatment with PB was carried out for 15 days subsequent to the treatment with sarin. **A:** After 3 months of sarin treatment; **B:** After 6 months of sarin treatment. The rats were treated with a single i.m. injection of 0.5xLD50 sarin, following which the rats in PB alone or sarin and PB were treated with 1.3 mg/kg PB (in water, oral by gavage), daily for 15 days. The control and sarin group rats were treated with water (oral by gavage). The rats from control and PB alone group were treated with i.m. injection of normal saline. The rats were maintained for a further period of 3 and 6 months. The animals were evaluated for beam-walk score, beam-walk time, incline plane and grip response. The data are presented as mean \pm SE, n=5. *Indicates statistically significant.

Figure 2: Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) activity 24 hrs after single i.m. treatment with 0.5x LD50 sarin, alone and in combination with PB. The treatment with PB was carried out for 15 days subsequent to the treatment with sarin. **A:** AChE activity in different brain regions; **B:** BChE activity in plasma. The rats were treated with a single i.m. injection of 0.5xLD50 sarin, following which the rats in PB alone or sarin and PB were treated with 1.3 mg/kg PB (in water, oral by gavage), daily for 15 days. The control and sarin group rats were treated with water (oral by gavage). The rats from control and PB alone group were treated with i.m. injection of normal saline. The rats were sacrificed 24 hrs after the treatment with sarin. Enzyme activity was determined as described in Materials and Methods. The control activity was 1.77 ± 0.31 cortex; 0.32 ± 0.04 brainstem; 0.55 ± 0.14

midbrain; 0.61 ± 0.04 cerebellum and 0.04 ± 0.01 , plasma expressed as μ moles substrate hydrolyzed/min/mg protein. The data are presented as mean + SE (% of control), n = 5.

* Indicates statistically significant.

Figure 3: Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) 3 months after a single i.m. treatment with 0.5x LD50 sarin. The treatment with PB was carried out for 15 days subsequent to the treatment with sarin. **A:** AChE activity in different brain regions; **B:** BChE activity in plasma. The rats were treated with a single i.m. injection of 0.5xLD50 sarin, following which the rats in PB alone or sarin and PB were treated with 1.3 mg/kg PB (in water, oral by gavage), daily for 15 days. The control and sarin group rats were treated with water (oral by gavage). The rats from control and PB alone group were treated with i.m. injection of normal saline. The rats were maintained for a further period of 3 months. Enzyme activity was determined as described in Materials and Methods. The control activity was 0.97 ± 0.25 cortex; 1.35 ± 0.02 brainstem; 1.41 ± 0.05 midbrain; 0.56 ± 0.02 cerebellum and 0.05 ± 0.001 , plasma expressed as μ moles substrate hydrolyzed/min/mg protein. The data are presented as mean + SE (% of control), n = 5. * Indicates statistically significant.

Figure 4: Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) 6 months after a single i.m. treatment with 0.5x LD50 sarin. The treatment with PB was carried out for 15 days subsequent to the treatment with sarin. **A:** AChE activity in different brain regions; **B:** BChE activity in plasma. The rats were treated with a single i.m. injection of 0.5xLD50 sarin, following which the rats in PB alone or sarin and PB were treated with 1.3 mg/kg PB (in water, oral by gavage),

daily for 15 days. The control and sarin group rats were treated with water (oral by gavage). The rats from control and PB alone group were treated with i.m. injection of normal saline. The rats were maintained for a further period of 6 months. Enzyme activity was determined as described in Materials and Methods. The control activity was 1.49 ± 0.25 cortex; 1.87 ± 0.03 brainstem; 1.18 ± 0.15 midbrain; 0.65 ± 0.03 cerebellum and 0.04 ± 0.001 , plasma expressed as μ moles substrate hydrolyzed/min/mg protein. The data are presented as mean + SE (% of control), n = 5. * Indicates statistically significant.

Table 1

ChE Activity (PB/sarin-24hr)
mean value (μ moles/min/mg protein)

Regions	name of the group							
	control		PB		SARIN		PB/SARIN	
	mean	SE	mean	SE	mean	SE	mean	SE
Cortex	1.77	0.31	1.45	0.51	1.3	0.31	1.13	0.11
Brainstem	0.32	0.04	0.5	0.11	0.66 *	0.1	0.71 *	0.09
Midbrain	0.55	0.14	0.84	0.05	1.19 *	0.15	0.89	0.18
Cerebellum	0.61	0.04	0.64	0.03	0.53	0.13	0.74	0.07
Plasma	0.04	0.01	0.04	0.01	0.08	0.01	0.07	0.01

ChE Activity (PB/sarin-24hr)
(% of control)

Regions	name of the group							
	control		PB		SARIN		PB/SARIN	
	% of control	SE	% of control	SE	% of control	SE	% of control	SE
Cortex	100	17.81	82.3	28.75	73.9	17.8	64.1	6.41
Brainstem	100	11.27	158.4	36.04	208.7 *	31.06	223.5 *	29.26
Midbrain	100	26.2	153.9	9.14	217.8 *	27.58	162.8	33.34
Cerebellum	100	7.29	105.3	4.81	86.6	20.75	121.4	11.28
Plasma	100	21.08	100.9	11.99	178.5	32.64	167.1	26.19

Table 2

ChE Activity (PB/sarin-3months)
mean value (μ moles/min/mg protein)

Regions	name of the group							
	control		PB		SARIN		PB/SARIN	
	mean	SE	mean	SE	mean	SE	mean	SE
Cortex	0.97	0.25	1.46	0.29	1.3	0.22	0.85	0.05
Brainstem	1.35	0.02	1.58 *	0.05	1.46 *	0.04	1.51 *	0.02
Midbrain	1.41	0.05	1.72 *	0.05	1.84 *	0.1	1.57	0.06
Cerebellum	0.56	0.02	0.67	0.065	0.72 *	0.048	0.6	0.041
Plasma	0.05	0.001	0.09 *	0.01	0.07	0.01	0.05	0.01

ChE Activity (PB/sarin-3months)
(% of control)

Regions	name of the group							
	control		PB		SARIN		PB/SARIN	
	% of control	SE	% of control	SE	% of control	SE	% of control	SE
Cortex	100	25.73	149.8	29.32	134.1	22.73	87.67	5.47
Brainstem	100	0.98	117.1 *	3.77	108.1 *	3.17	111.6 *	1.12
Midbrain	100	3.78	121.58 *	3.82	130.1 *	7.46	110.96	4.32
Cerebellum	100	4.21	119.6	11.64	129.16 *	8.62	108.1	7.4
Plasma	100	8.96	182.9 *	17.22	129	12.07	101.1	24.49

Table 3

ChE Activity (PB/sarin-6months)
mean value (μ moles/min/mg protein)

Regions	name of the group							
	control		PB		SARIN		PB/SARIN	
	mean	SE	mean	SE	mean	SE	mean	SE
Cortex	1.49	0.25	1.12	0.24	1.39	0.34	0.73	0.07
Brainstem	1.87	0.03	2.1 *	0.06	2.29 *	0.07	2.06	0.18
Midbrain	1.18	0.15	1.46	0.056	1.37	0.027	1.48	0.039
Cerebellum	0.65	0.03	0.68	0.043	0.63	0.02	0.59	0.02
Plasma	0.04	0.001	0.08	0.01	0.04	0.01	0.03	0.01

ChE Activity (PB/sarin-6months)
(% of control)

Regions	name of the group							
	control		PB		SARIN		PB/SARIN	
	% of control	SE	% of control	SE	% of control	SE	% of control	SE
Cortex	100	17.24	75.21	16.2	93.32	22.57	49.62	4.19
Brainstem	100	1.6	112.97 *	3.29	123.02 *	3.76	110.58	9.98
Midbrain	100	13.04	123.11	4.75	115.95	2.33	125.35	3.34
Cerebellum	100	3.99	104.19	6.62	95.79	3.02	89.9	3.48
Plasma	100	7.54	180.9	32.09	95.7	22.71	79.4	17.22

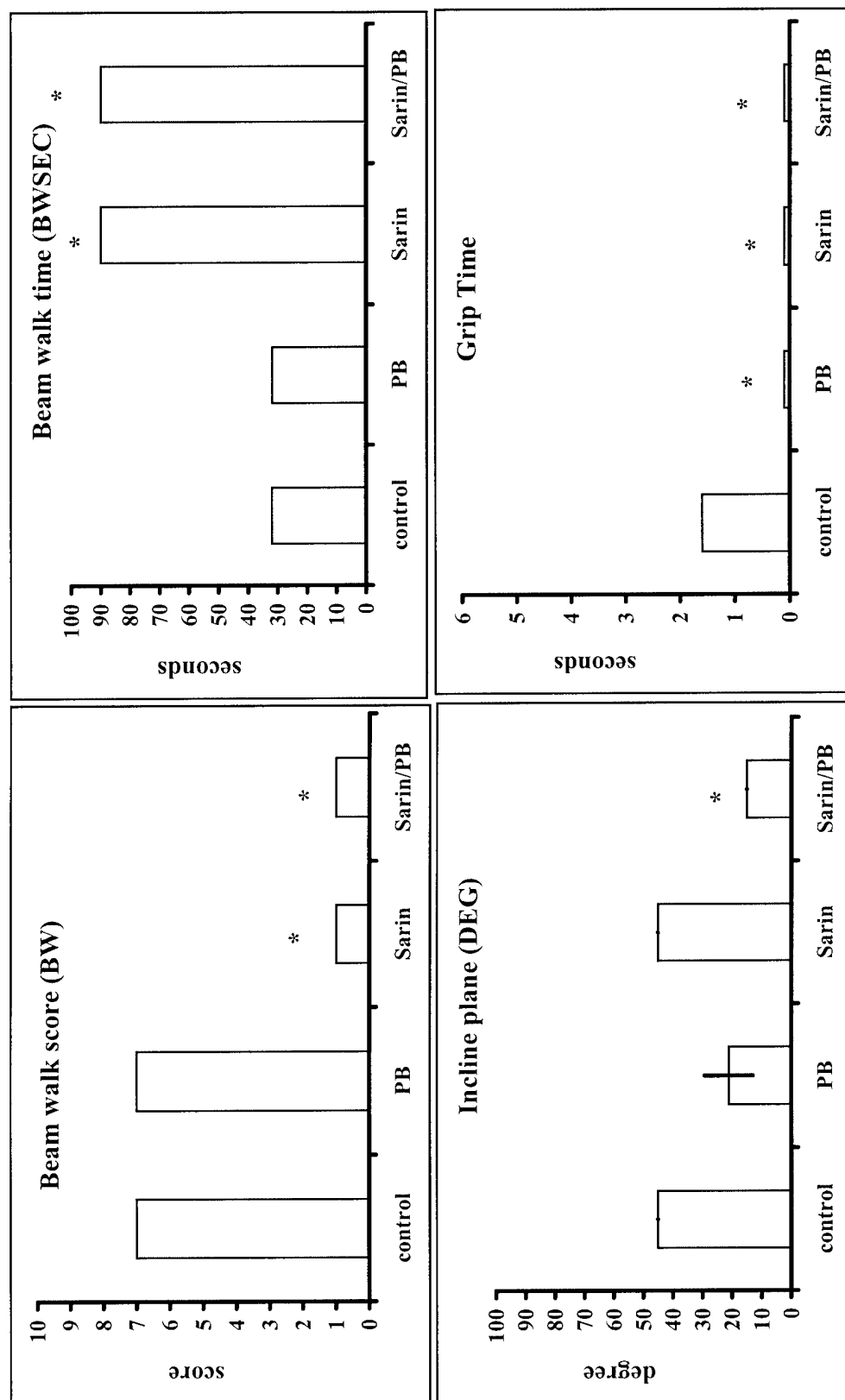


Figure 1 A : Behavioral studies (3 months)

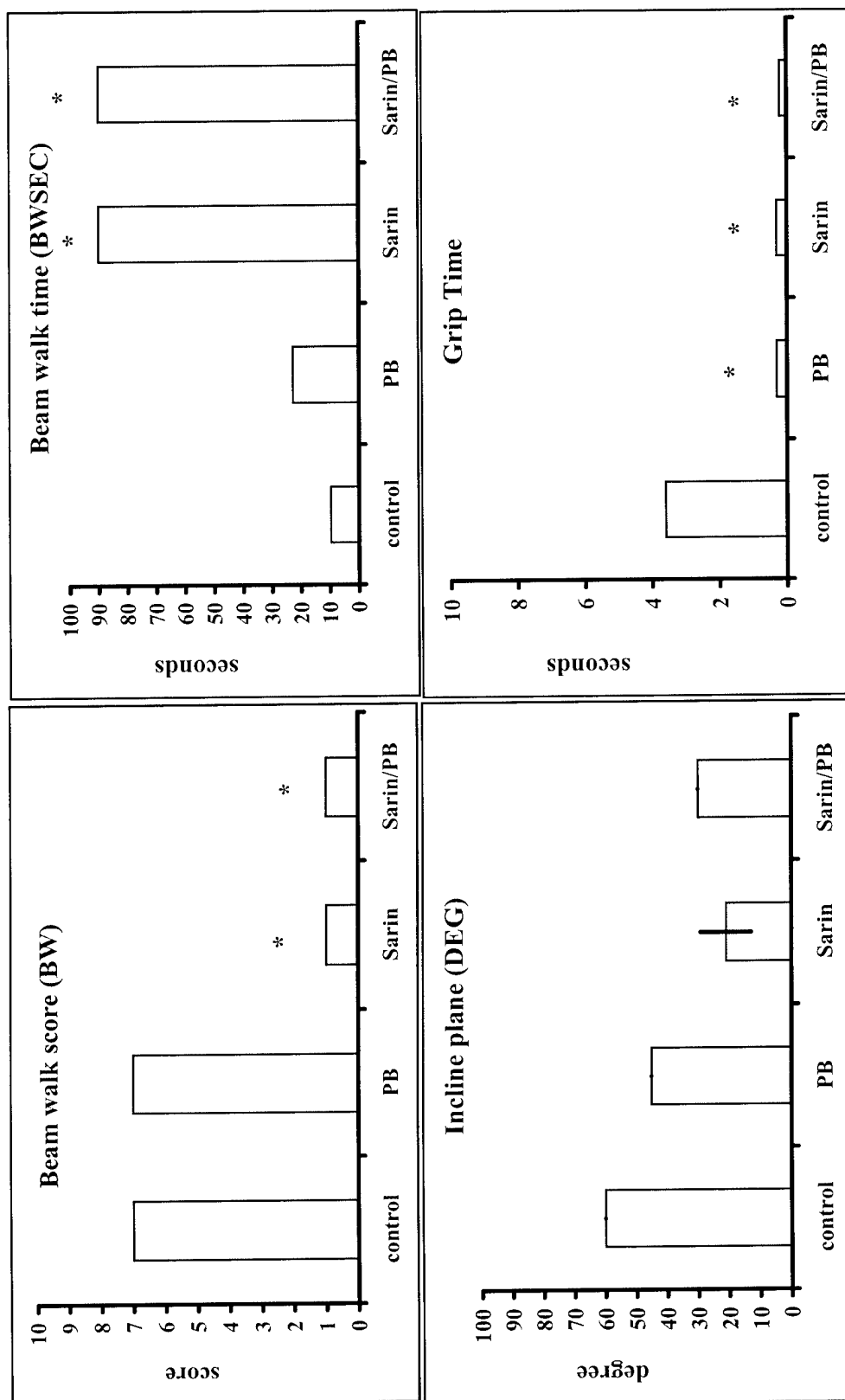


Figure 1 B : Behavioral studies (6 months)

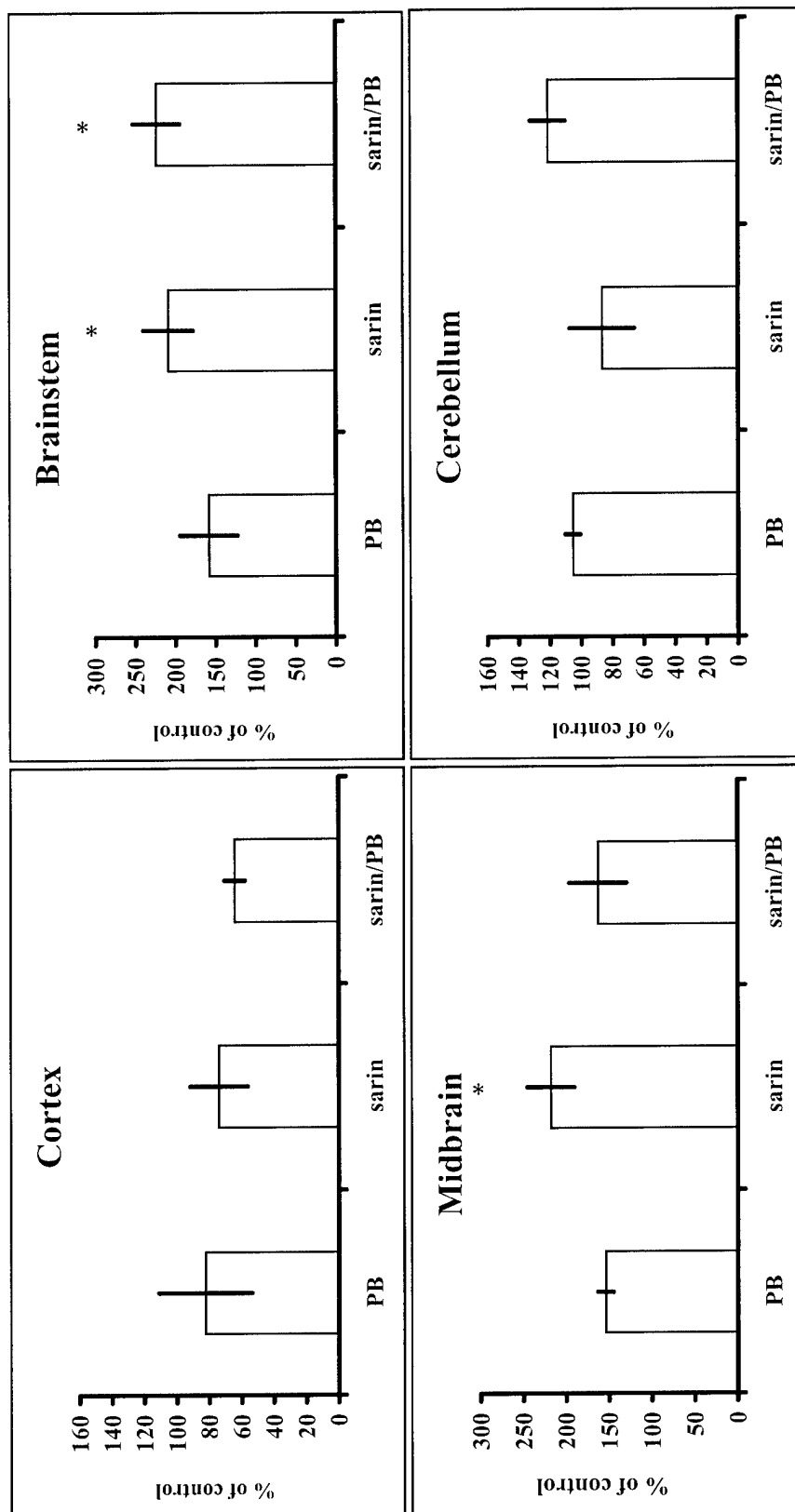


Figure 2 A : Brain regional AChE activity (Sarin/PB -24hr)

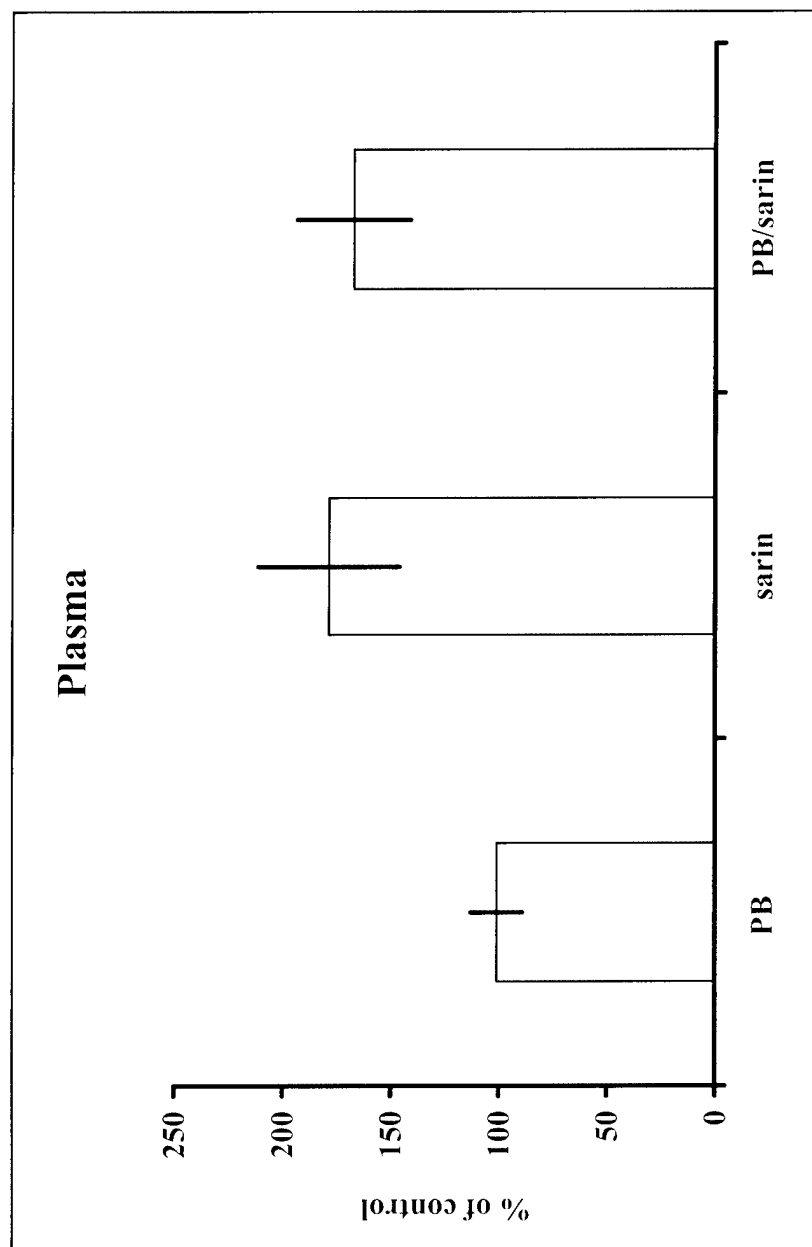


Figure 2 B : Plasma BChE activity (Sarin/PB -24hr)

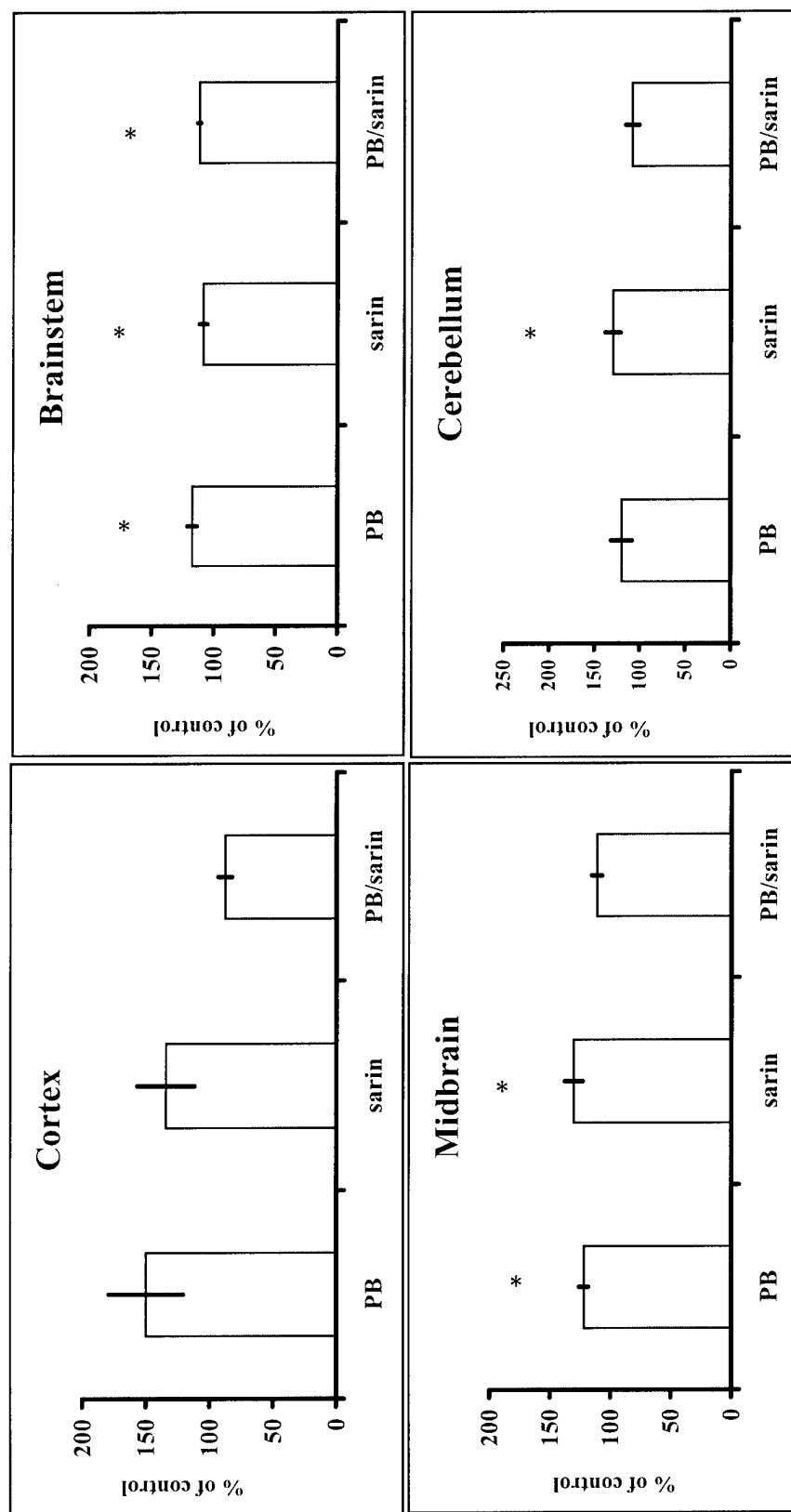


Figure 3 A : Brain regional AChE activity (Sarin/PB- 3months)

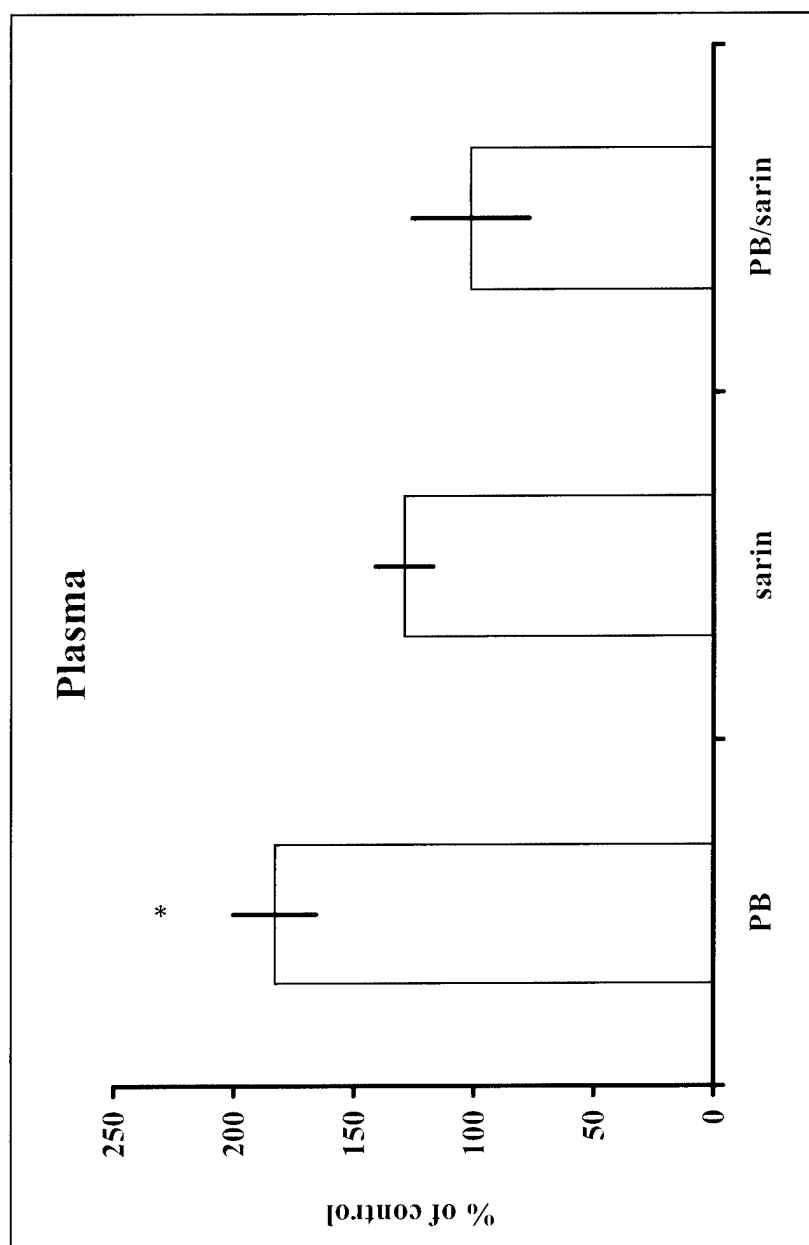


Figure 3 B : Plasma BChE activity (Sarin/PB- 3months)

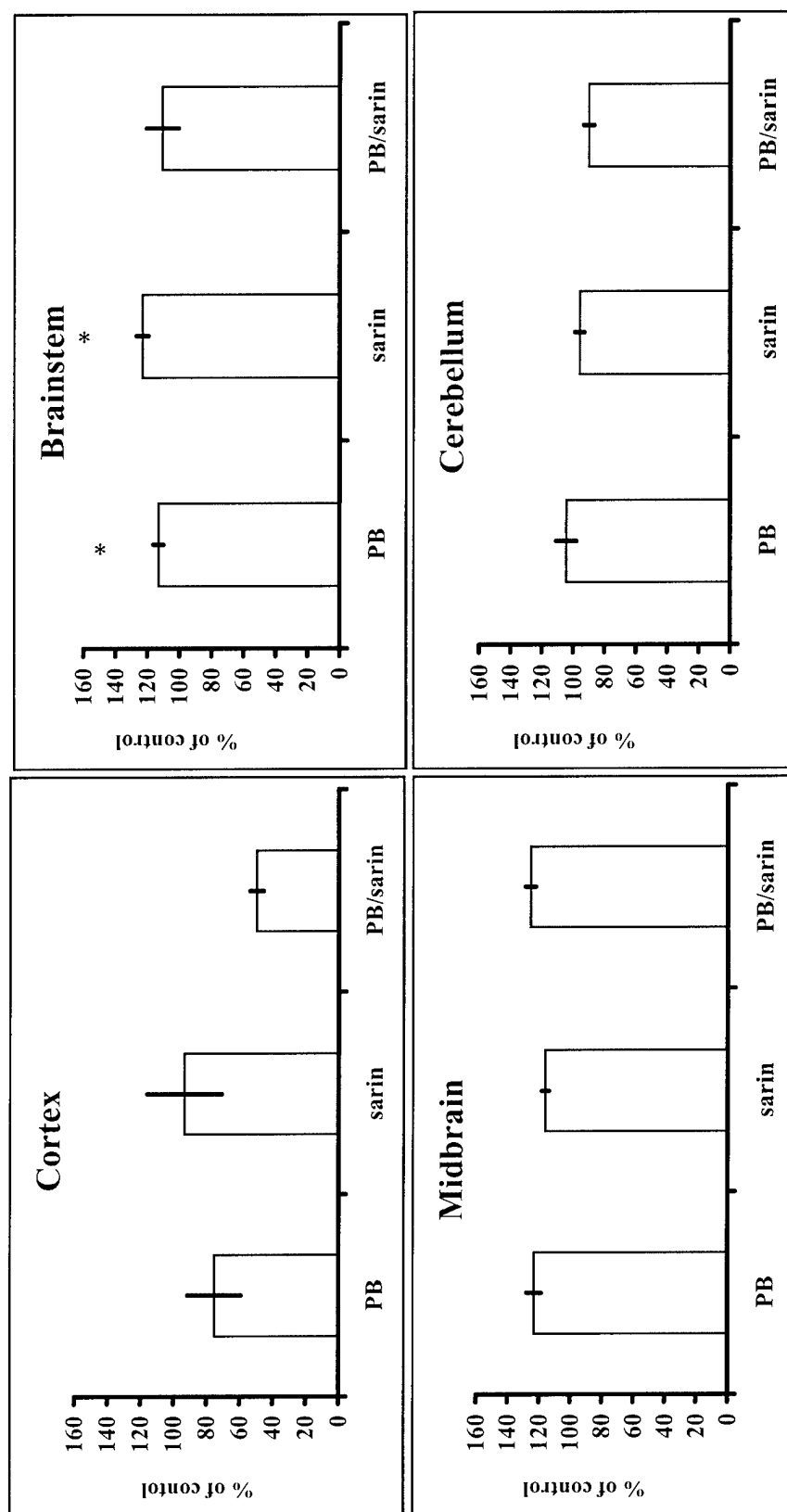


Figure 4 A : Brain regional AChE activity (Sarin/PB- 6 months)

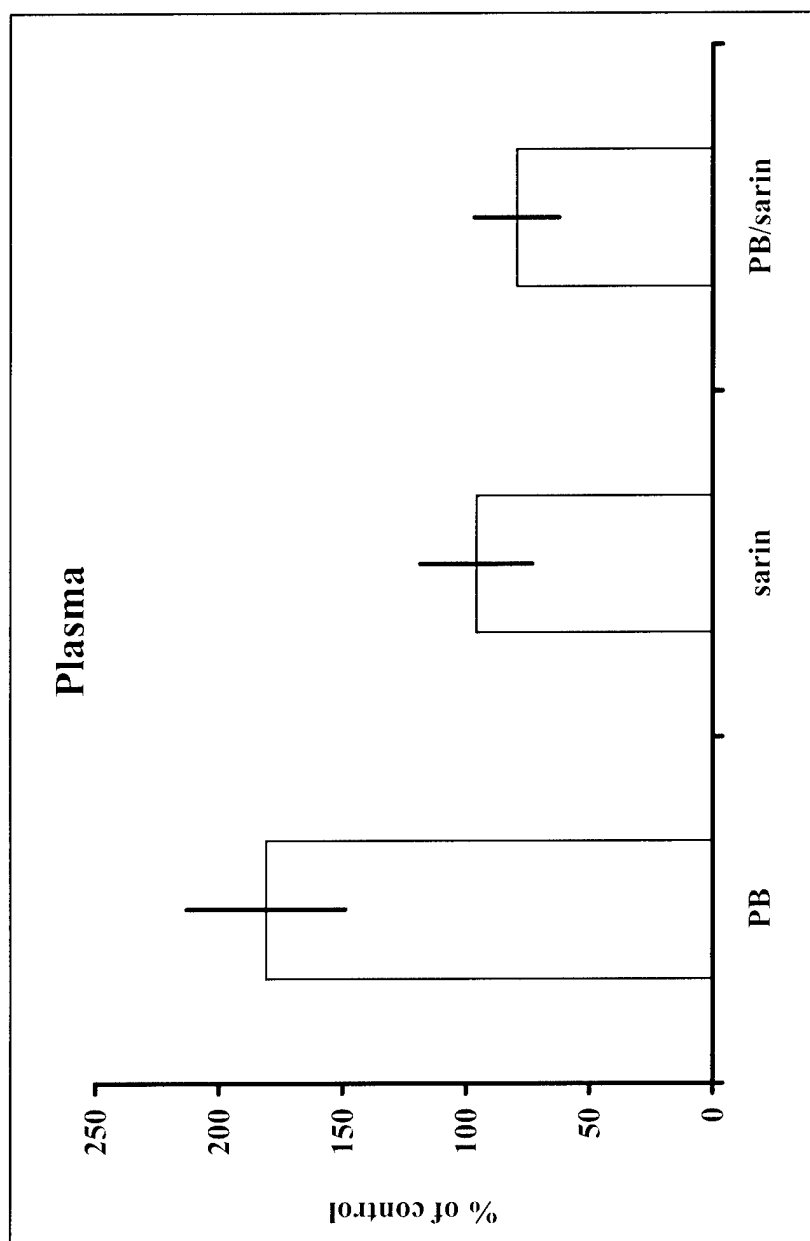


Figure 4 B : Plasma BChE activity (Sarin/PB- 6 months)

Specific Aim 6. Effect of stress and prophylactic treatment with PB on sub-clinical sarin exposure.

Rationale: Possible health effects of Low-level exposure to sarin during the PGW might have been compounded due to a variety of factors. The main modulatory factors that are thought to have played important role in PGW illness are variety of different chemicals, including PB, and stress. The objective of this specific aim is to study the long-term health effects of low-dose sarin exposure in combination with PB and stress.

Materials and Methods: Male Sprague-Dawley rats (225-250g) were obtained from Zivic-Miller Laboratories, Allison Park, PA. The animals were housed in Duke University Medical Center vivarium at 21-23 °C and on a 12-h dark-light cycle. The animals were allowed food and water *ad libitum*. All the treatments and procedures on the animals were carried out strictly according to the recommended guidelines by the Duke University Institutional Animal Care and Use Committee and U.S. Department of Defense.

Treatment of Animals and Tissue Retrieval

Animals were randomly divided into following groups of 5 animals each for each time point of the study.

Control: The rats in this group were handled without subjecting them to the stress daily for 15 days, and then on the last day treated with single i.m. injection of saline.

Stress alone: The rats were subjected to daily immobilization stress by placing them in Plexiglas restrainer tube for 5 minutes, daily for 15 days.

PB alone: The rats were treated with 1.3 mg/kg PB (in water, oral by gavage), daily for 15 days.

Sarin alone: The rats were treated with single i.m. injection of 0.5xLD50 sarin.

Exposure to PB and sarin: The rats were treated with 1.3 mg/kg PB (in water, oral by gavage), daily for 15 days. On the 15th day following treatment with PB, the rats were treated with single i.m. injection of 0.5xLD50 sarin.

Exposure to stress and sarin: The rats were subjected to daily immobilization stress by placing them in Plexiglas restrainer tube for 5 minutes, daily for 15 days. On day 15 following completion of the stress, the rats were treated with single i.m. injection of 0.5xLD50 sarin.

Exposure to stress and PB: The rats were subjected to daily immobilization stress by placing them in Plexiglas restrainer tube for 5 minutes, followed by within 30 minutes treatment with 1.3 mg/kg PB (in water, oral by gavage), daily for 15 days.

Exposure to stress, PB and sarin: The rats were subjected to daily immobilization stress by placing them in Plexiglas restrainer tube for 5 minutes, followed by within 30 minutes treatment with 1.3 mg/kg PB (in water, oral by gavage), daily for 15 days. On day 15th following the treatment with the last dose of PB, the rats were treated with single i.m. injection of 0.5xLD50 sarin.

At 24 hrs, 3 and 6 month following treatment with sarin, the rats were anesthetized with ketamine/xylazine (100mg/kg ketamine, 15mg/kg xylazine) and blood was drawn in heparinized syringe. Brains were removed and washed thoroughly with ice cold normal saline to remove blood. Brain regions, cortex, midbrain, cerebellum and brainstem were dissected on ice and snap frozen in liquid nitrogen. Plasma was separated and frozen at -80 °C for enzyme studies.

Behavioral Testing Battery:

The behavioral tests employed in these studies evaluate sensorimotor reflexes, motor strength, and coordination. All behavioral testing was performed by an observer blind to the animal's treatment status and was carried out in a sound-proof room with subdued lighting (less than 10.76 lumens/m², ambient light) between 7 and 11:30 AM. Behavioral testing was carried out at 3 and 6 months following single i.m. injection of 0.5xLD₅₀ sarin.

Reflexes:

Postural reflexes (Bederson, et al, 1986; Markgraf, et al, 1992), visual, tactile and proprioceptive forelimb placing responses (Markgraf et al, 1992) and orienting to vibrissae touch (Whishaw et al, 1985) were carried out as described by Abou-Donia et al (2001).

Inclined Plane:

Description

Rats were placed on a flat plane in the horizontal position, with the head facing the side of the board to be raised (Yonemori et al, 1998) and Abou-Donia et al (2002).

Scoring: The angle that the rat begins to slip downward was recorded. The results of the two trials were averaged for each testing session.

Forepaw Grip Time:

Description

The rat's forepaw strength was assessed by having them grip a 5 mm diameter wood dowel that was held horizontally and raised so that the rat supports its body weight as described by Andersen et al (1991) and Abou-Donia et al (2002).

Scoring: Time to release grip was recorded in seconds. The results of the two trials were averaged for each testing session.

Beam-Walking:

Description

The testing apparatus was a 2.5 X 122 cm wooden beam elevated 75.5 cm above the floor with wooden supports as described by Goldstein (1993) and Abou-Donia et al (2002).

Scoring: Beam-walking ability was measured with a seven point scoring system scale as previously described by Goldstein (1993 & 1995) as detailed by Abou-Donia et al (2002).

Statistical Analyses

Comparisons across treatment groups for postural reflexes, limb placing, and vibrissae touch orientation were analyzed with non-parametric analysis of variance (Kruskal-Wallis test). Data for the remaining behavioral tests were compared among groups by one-way or two-way repeated measures ANOVA as appropriate. If a significant difference was found, Fisher's LSD tests were applied to permit post-hoc, pair-wise comparisons. A two-tailed P value of <0.05 was considered statistically significant.

Cholinesterase determination:

Activities of AChE in brain regions and BChE in plasma were determined according to the method of Ellman et al, (1961), modified for assay in a Molecular Devices UV Max Kinetic Microplate Reader, as previously described (Abou-Donia et al, 1996, 2001, 2002). Protein concentration was determined by BCA method according to

Smith et al, (1985). The enzyme activities are expressed as μ mole substrate hydrolyzed/min/mg protein.

Statistical Analysis:

The results were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test. P value <0.05 was considered significant.

Results and conclusions: The data are presented in appendix 6. Neurobehavioral evaluations for sensorimotor performance are presented in Fig 1 A& B for 3 and 6 months, respectively. Beam-walk score was significantly impaired at 3 months in the animals treated with stress and a combination of stress with PB. Treatment with stress and PB alone and a combination of stress with either PB or sarin resulted in significant impairments at 6 month. For beam-walk time, incline plane, and forepaw grip there was a significant impairment in all the treated groups and at 3 and 6 month time periods. However, there was no additive effect when the rats were exposed to PB, sarin and stress in combination.

Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) are presented in Fig (2–4) for 24 hrs, 3 months and 6 months respectively. Data presented in Figure 2 show that stress caused a significant increase in brainstem and midbrain AChE activity (~ 138 and 127% of control, respectively). Treatment with a combination of stress and PB and stress in combination with PB and sarin resulted in a significant increase in midbrain AChE activity (~ 125 and 118% of control). Brain regional AChE and plasma BChE activity from 3 month group of rats are presented in Fig 3. There was a significant increase in cortex AChE activity ($\sim 159\%$ of control) following treatment with sarin alone ($\sim 159\%$ of control), stress alone

(~202% of control), and a combination of stress and PB (~188% of control). A significant increase in cortex AChE activity (~202% of control) and a significant decrease in AChE activity in the brainstem (~59% of control) was observed in the rats treated with stress and PB in combination. Figure 4 demonstrates the brain regional AChE and plasma BChE activity 6 months after sarin treatment. There was a significant increase in midbrain AChE activity in the animals treated with PB alone (~119% of control), stress alone (~128% of control) and stress and PB in combination (~136% of control) and a significant decrease in stress in combination with sarin (~56% of control). The cerebellum AChE activity showed an increase in the rats treated with sarin alone (~124% of control) or stress alone (~130% of control) or a combination of stress and sarin (~141% of control) or stress and PB (~148% of control). Exposure to a combination of sarin, PB and stress resulted in a significant increase in AChE activity in brainstem (120% of control), midbrain (~133% of control) and cerebellum (~137% of control).

Figure Legends:

Figure 1: Sensorimotor performance following a single i.m. treatment with 0.5x LD50 sarin, alone or in combination with pyridostigmine bromide (PB). **A:** After 3 month of sarin treatment; **B:** After 6 month of sarin treatment. The rats were treated with 1.3 mg/kg PB (in water, oral by gavage), daily for 15 days or subjected to immobilization stress by placing them in Plexiglas restrainer for 5 minutes for 15 days. The animals treated with PB and stress in combination were treated with stress and then treated with PB. The control and sarin group rats were treated with water (oral by gavage). On day 15, following the last treatment with PB and stress, the rats were treated with a single i.m. injection of 0.5xLD50 sarin. The rats from control and PB and stress alone or a combination of PB and stress groups were treated with i.m. injection of normal saline. The rats were maintained for a further period of 3 and 6 months. The animals were evaluated for beam-walk score, beam-walk time, incline plane and grip response. The data are presented as mean \pm SE, n=5. *Indicates statistically significant.

Figure 2: Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) activity 24 hrs after single i.m. treatment with 0.5x LD50 sarin, alone and in combination with PB. **A:** AChE activity in different brain regions; **B:** BChE activity in plasma. The rats were treated with 1.3 mg/kg PB (in water, oral by gavage), daily for 15 days or subjected to immobilization stress by placing them in Plexiglas restrainer for 5 minutes for 15 days. The animals treated with PB and stress in combination were treated with stress and then treated with PB. The control and sarin group rats were treated with water (oral by gavage). On day 15, following the last treatment with PB and stress, the rats were treated with a single i.m. injection of

0.5xLD50 sarin. The rats from control and PB and stress alone or a combination of PB and stress groups were treated with i.m. injection of normal saline. The rats were sacrificed 24 hrs after the treatment with sarin. Enzyme activity was determined as described in Materials and Methods. The control activity was 0.79 ± 0.11 cortex; 1.39 ± 0.06 brainstem; 1.39 ± 0.06 midbrain; 0.73 ± 0.04 cerebellum and 0.03 ± 0.001 , plasma expressed as μ moles substrate hydrolyzed/min/mg protein. The data are presented as mean + SE (% of control), n = 5. * Indicates statistically significant.

Figure 3: Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) 3 months after a single i.m. treatment with 0.5x LD50 sarin. **A:** AChE activity in different brain regions; **B:** BChE activity in plasma. The rats were treated with 1.3 mg/kg PB (in water, oral by gavage), daily for 15 days or subjected to immobilization stress by placing them in Plexiglas restrainer for 5 minutes for 15 days. The animals treated with PB and stress in combination were treated with stress and then treated with PB. The control and sarin group rats were treated with water (oral by gavage). On day 15, following the last treatment with PB and stress, the rats were treated with a single i.m. injection of 0.5xLD50 sarin. The rats from control and PB and stress alone or a combination of PB and stress groups were treated with i.m. injection of normal saline. The rats were maintained for a further period of 3 months. Enzyme activity was determined as described in Materials and Methods. The control activity was 0.88 ± 0.06 cortex; 2.01 ± 0.16 brainstem; 1.38 ± 0.08 midbrain; 0.48 ± 0.02 cerebellum and 0.039 ± 0.001 , plasma expressed as μ moles substrate hydrolyzed/min/mg protein. The data are presented as mean + SE (% of control), n = 5. * Indicates statistically significant.

Figure 4: Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) 6 months after a single i.m. treatment with 0.5x LD50 sarin. **A:** AChE activity in different brain regions; **B:** BChE activity in plasma. The rats were treated with 1.3 mg/kg PB (in water, oral by gavage), daily for 15 days or subjected to immobilization stress by placing them in Plexiglas restrainer for 5 minutes for 15 days. The animals treated with PB and stress in combination were treated with stress and then treated with PB. The control and sarin group rats were treated with water (oral by gavage). On day 15, following the last treatment with PB and stress, the rats were treated with a single i.m. injection of 0.5xLD50 sarin. The rats from control and PB and stress alone or a combination of PB and stress groups were treated with i.m. injection of normal saline. The rats were maintained for a further period of 6 months. Enzyme activity was determined as described in Materials and Methods. The control activity was 1.27 ± 0.28 cortex; 1.48 ± 0.09 brainstem; 1.20 ± 0.05 midbrain; 0.5 ± 0.02 cerebellum and 0.06 ± 0.01 , plasma expressed as μ moles substrate hydrolyzed/min/mg protein. The data are presented as mean + SE (% of control), n = 5. * Indicates statistically significant.

Table 1

ChE Activity (PB/stress/sarin-24hr -experiment)
mean value (μ moles/min/mg protein)

Regions	name of the group											
	CONTROL		PB		Stress		Sarin		Stress/Sarin		PB/Stress	
	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
Cortex	0.79	0.11	0.98	0.17	0.91	0.12	0.6	0.13	0.72	0.07	0.88	0.08
Brainstem	1.39	0.21	1.79	0.05	1.92 *	0.06	1.88	0.1	1.3	0.21	1.75	0.05
Midbrain	1.39	0.06	1.36	0.05	1.77 *	0.09	1.56	0.08	1.56	0.06	1.74 *	0.12
Cerebellum	0.73	0.04	0.73	0.03	0.79	0.06	0.72	0.07	0.71	0.03	0.65	0.03
Plasma	0.03	0.001	0.02	0.001	0.03	0.01	0.03	0.001	0.03	0.01	0.03	0.001

ChE Activity (PB/stress/sarin-24hr-experiment)
(% of control)

Regions	name of the group											
	CONTROL		PB		Stress		Sarin		Stress/Sarin		PB/Stress	
	(% cont)	SE	(% cont)	SE	(% cont)	SE	(% cont)	SE	(% cont)	SE	(% cont)	SE
Cortex	100	13.4	123.9	21.9	115.6	15.1	76.7	15.9	91.1	9.1	111.5	12.9
Brainstem	100	14.9	128.4	3.4	138.2 *	4.6	135.5	7.4	93.6	15.2	125.9	3.7
Midbrain	100	4.0	98	3.6	127.3 *	6.8	112.7	6.1	112.7	4.4	125.5 *	8.6
Cerebellum	100	5.9	98.9	3.4	107.9	8.0	98.7	10.1	96.2	3.6	89.2	4.1
Plasma	100	16.5	85.2	7.9	104.1	21.7	113.3	14.4	113.7	19.1	108.3	4.8

Table 2

ChE Activity (PB/stress/sarin-3months -experiment)
mean value (μ moles/min/mg protein)

Regions	name of the group											
	CONTROL		PB		Stress		Sarin		Stress/Sarin		PB/Stress	
	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
Cortex	0.88	0.065	1.46	0.4	1.78 *	0.46	1.40 *	0.24	0.94	0.16	1.65 *	0.26
Brainstem	2.01	0.16	1.63	0.1	2.65	0.58	1.61	0.18	2.13	0.2	1.32 *	0.19
Midbrain	1.38	0.08	1.68	0.1	1.59	0.08	1.4	0.1	1.44	0.05	1.5	0.14
Cerebellum	0.48	0.02	0.42	0.02	0.5	0.037	0.48	0.3	0.49	0.03	0.51	0.02
Plasma	0.039	0.001	0.036	0.001	0.031	0.005	0.038	0.01	0.039	0.01	0.041	0.01
											0.029	0.01
											0.056 *	0.00

ChE Activity (PB/stress/sarin-3months-experiment)
(% of control)

Regions	name of the group											
	CONTROL		PB		Stress		Sarin		Stress/Sarin		PB/Stress	
	(% cont)	SE	(% cont)	SE	(% cont)	SE	(% cont)	SE	(% cont)	SE	(% cont)	SE
Cortex	100	7.4	166.1	51.0	202.5 *	52.9	159.9 *	27.4	107.1	18.6	188.2 *	29.7
Brainstem	100	8.0	81.2	5.2	104.7	12.8	79.9	8.7	106.1	9.7	65.59 *	9.3
Midbrain	100	6.0	122.6	7.4	115.9	5.9	102.6	7.3	105.4	3.7	110.2	10.5
Cerebellum	100	3.9	87.8	4.5	102.8	7.8	100.0	6.5	101.7	5.4	104.8	4.9
Plasma	100	3.4	91.6	5.0	79.22	20.6	98.2	20.1	101.2	21.2	104.6	14.9
											73.7	19.3
											144.9 *	9.0

Table 3

ChE Activity (PB/stress/sarin-6months -experiment)
mean value (μ moles/min/mg protein)

Regions	name of the group											
	CONTROL		PB		Stress		Sarin		PB/Stress		PB/Sarin	
	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
Cortex	1.27	0.28	0.69	0.09	0.84	0.23	1.33	0.43	1.20	0.45	0.73	0.17
Brainstem	1.48	0.09	1.42	0.14	1.21	0.19	1.48	0.07	1.71	0.12	1.48	0.10
Midbrain	1.20	0.05	1.41 *	0.07	1.52 *	0.09	1.59	0.27	1.62 *	0.23	1.37	0.13
Cerebellum	0.50	0.02	0.45	0.06	0.65 *	0.04	0.62 *	0.03	0.73 *	0.04	0.53	0.05
Plasma	0.06	0.01	0.08	0.01	0.06	0.01	0.07	0.01	0.04	0.01	0.04	0.01

ChE Activity (PB/stress/sarin-6months-experiment)
(% of control)

Regions	name of the group											
	CONTROL		PB		Stress		Sarin		PB/Stress		PB/Sarin	
	(% cont)	SE	(% cont)	SE	(% cont)	SE	(% cont)	SE	(% cont)	SE	(% cont)	SE
Cortex	100.0	22.4	54.2	7.0	66.7	18.1	105.2	34.6	94.8	35.6	57.8	13.0
Brainstem	100.0	6.3	96.1	9.3	81.9	12.6	99.6	4.9	115.7	8.2	100.3	6.8
Midbrain	100.0	4.3	119.1 *	6.0	128.1 *	7.5	134.7	22.6	136.7 *	1.9	115.3	10.6
Cerebellum	100.0	4.3	91.3	12.7	130.7 *	7.9	124.8 *	6.5	148.2 *	7.4	106.2	10.7
Plasma	100.0	14.4	135.3	10.1	101.3	19.5	117.9	9.4	66.4	14.6	60.0	14.6

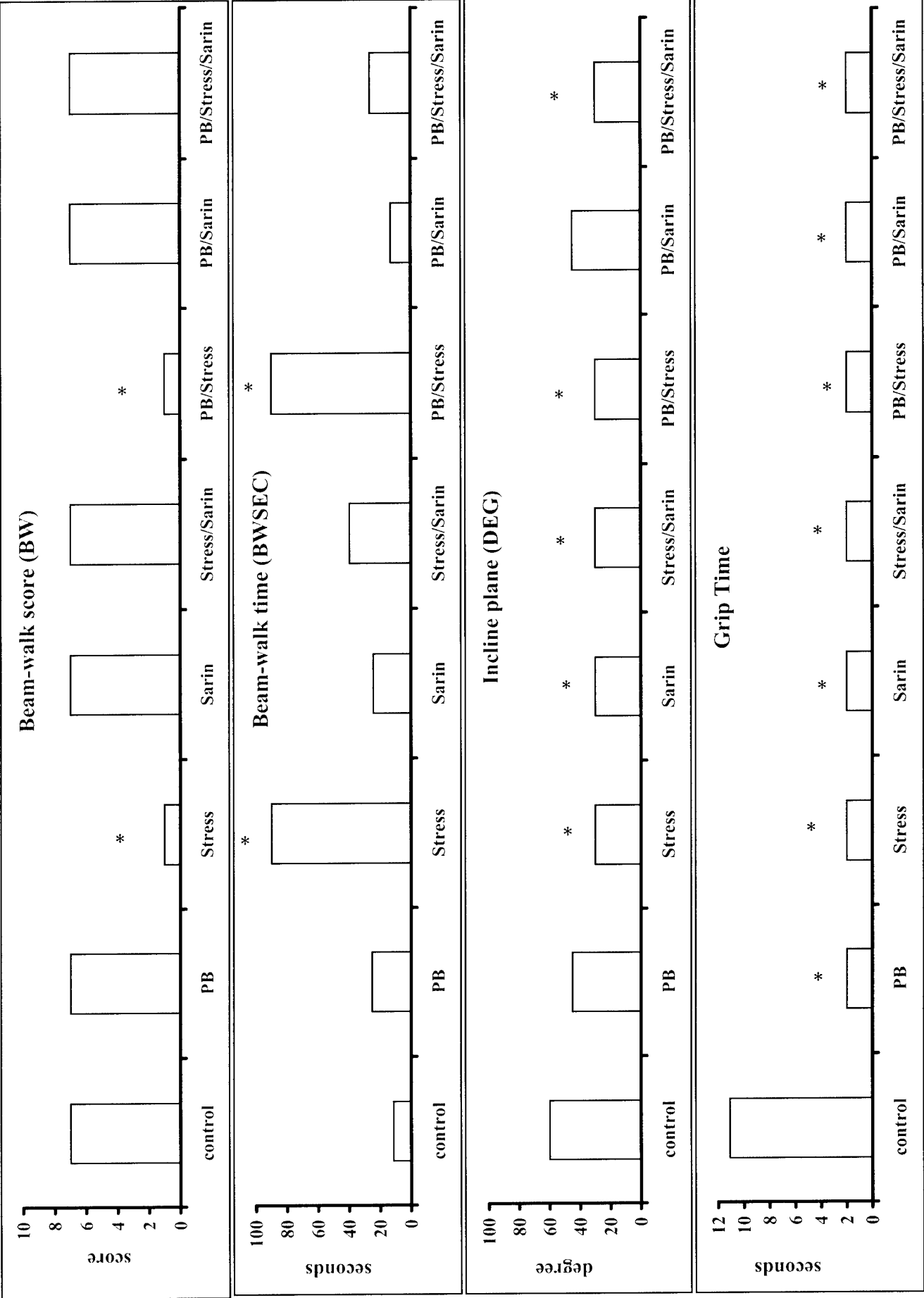


Figure 1 A : Behavioral studies (3months)

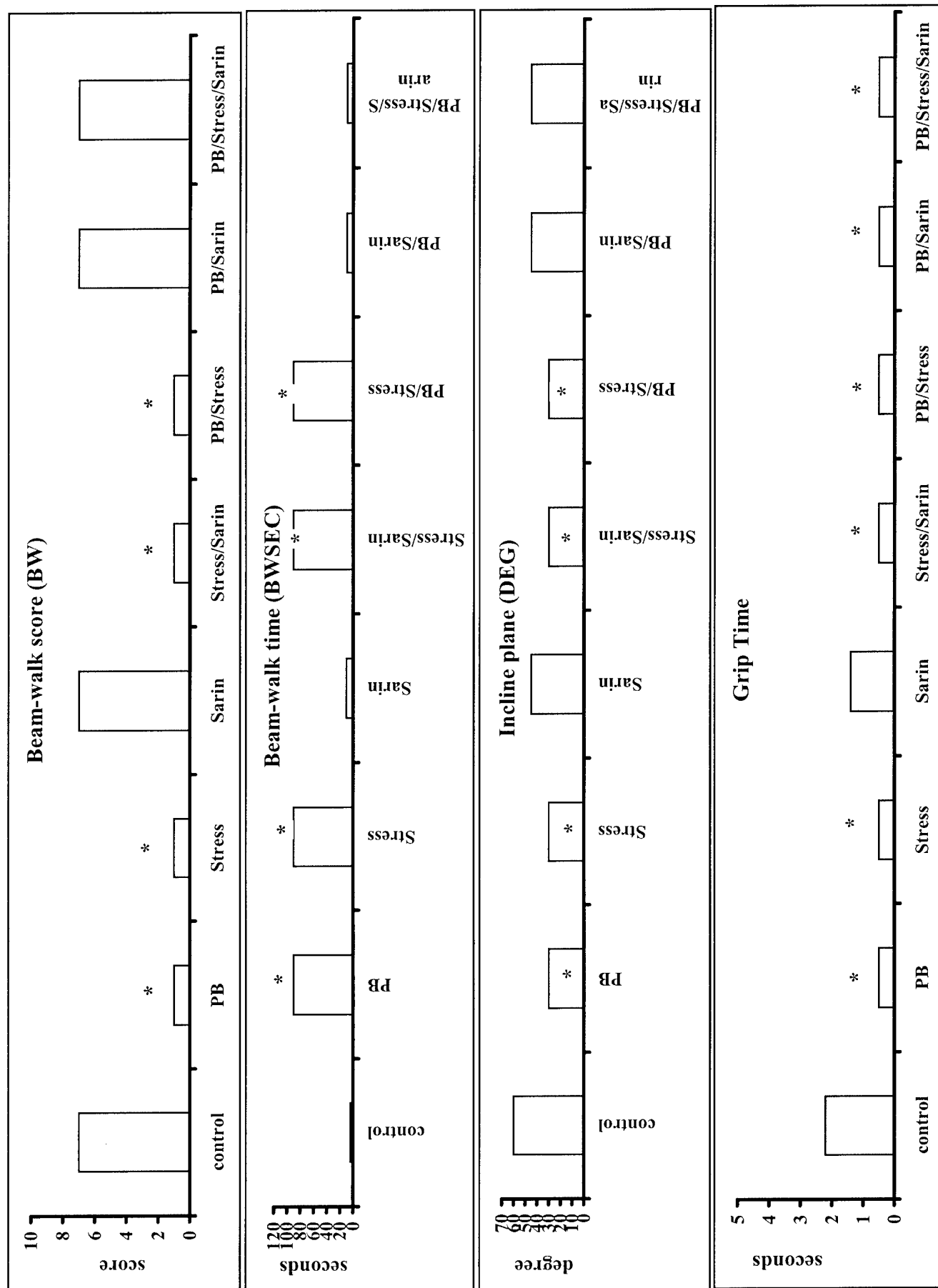


Figure 1 B : Behavioral studies (6 months)

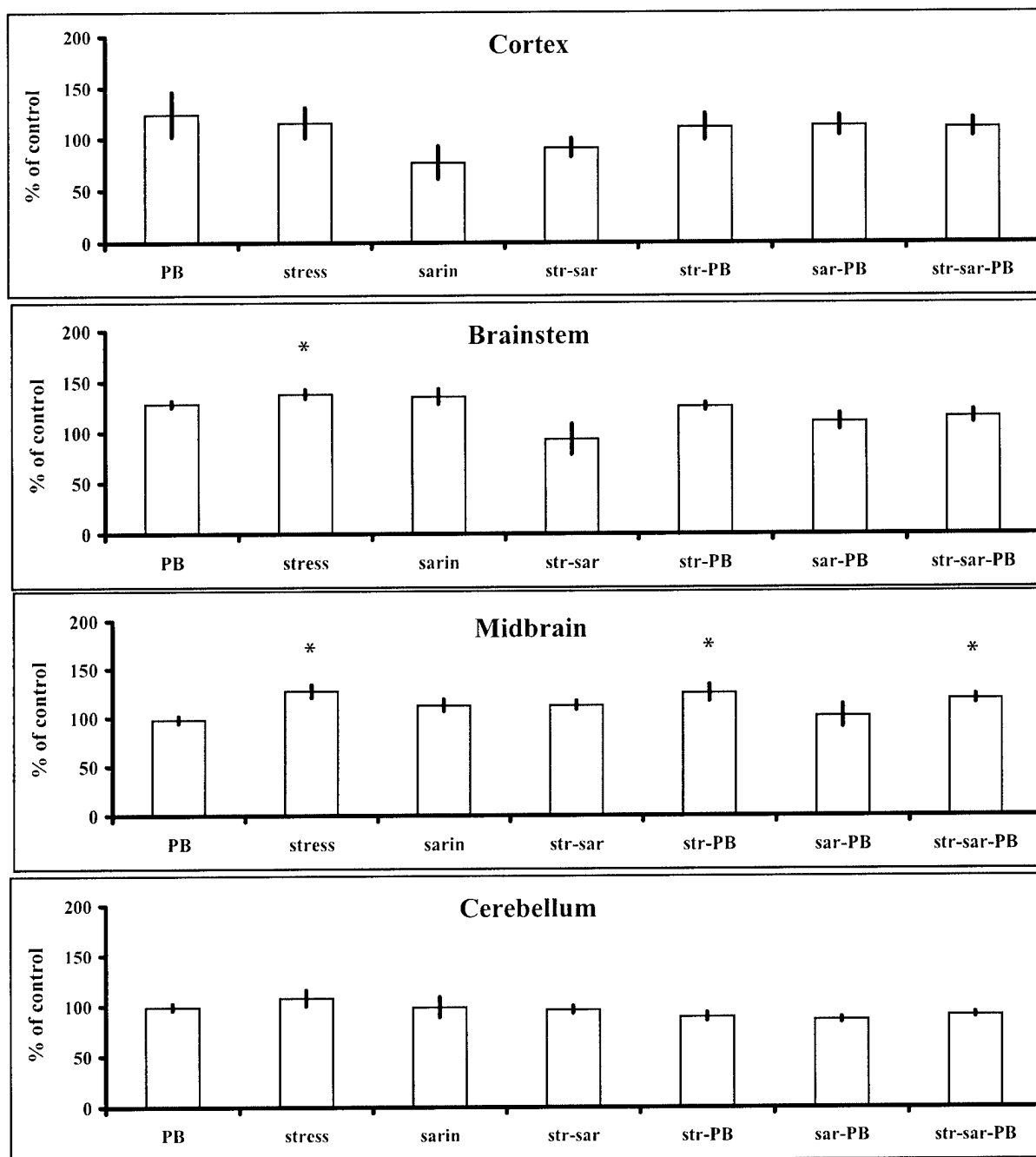


Figure 2 A : Brain regional AChE activity (PB/sarin/stress -24hr)

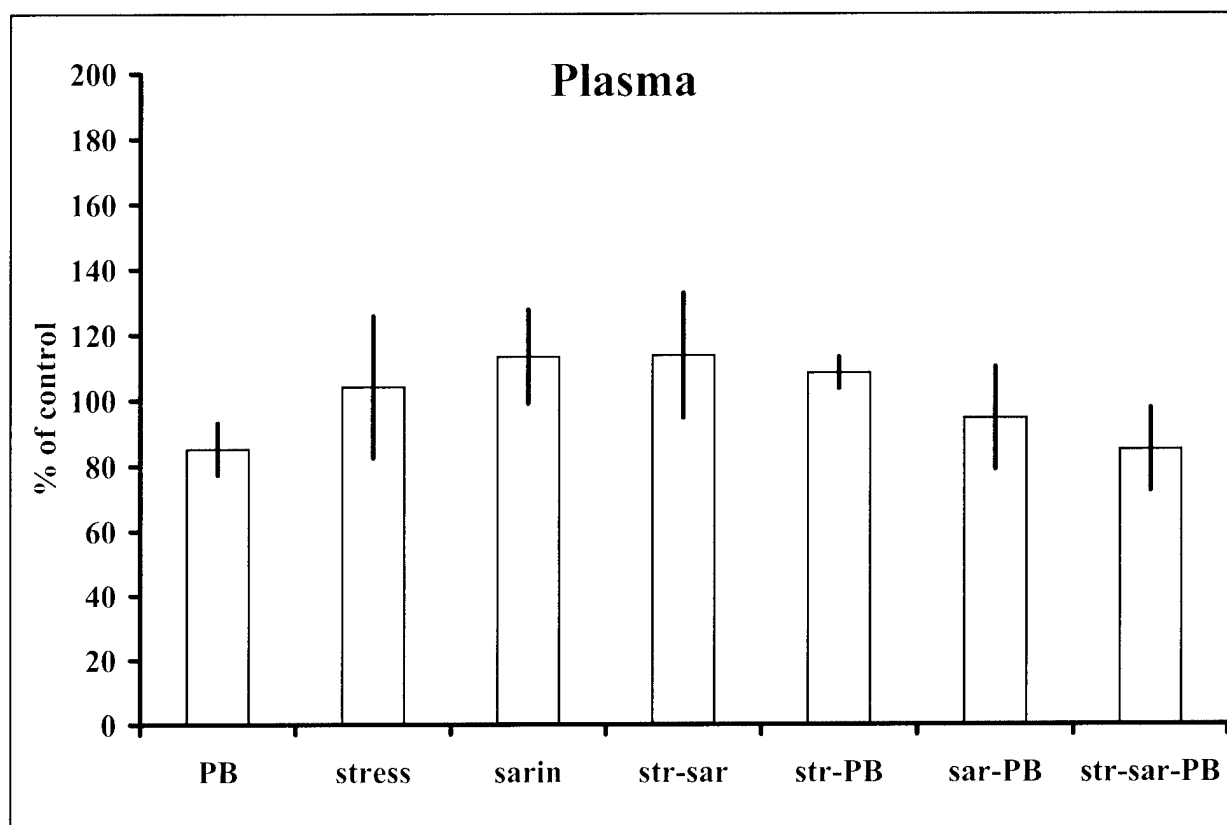


Figure 2 B : Plasma BChE activity (PB/sarin/stress -24hr)

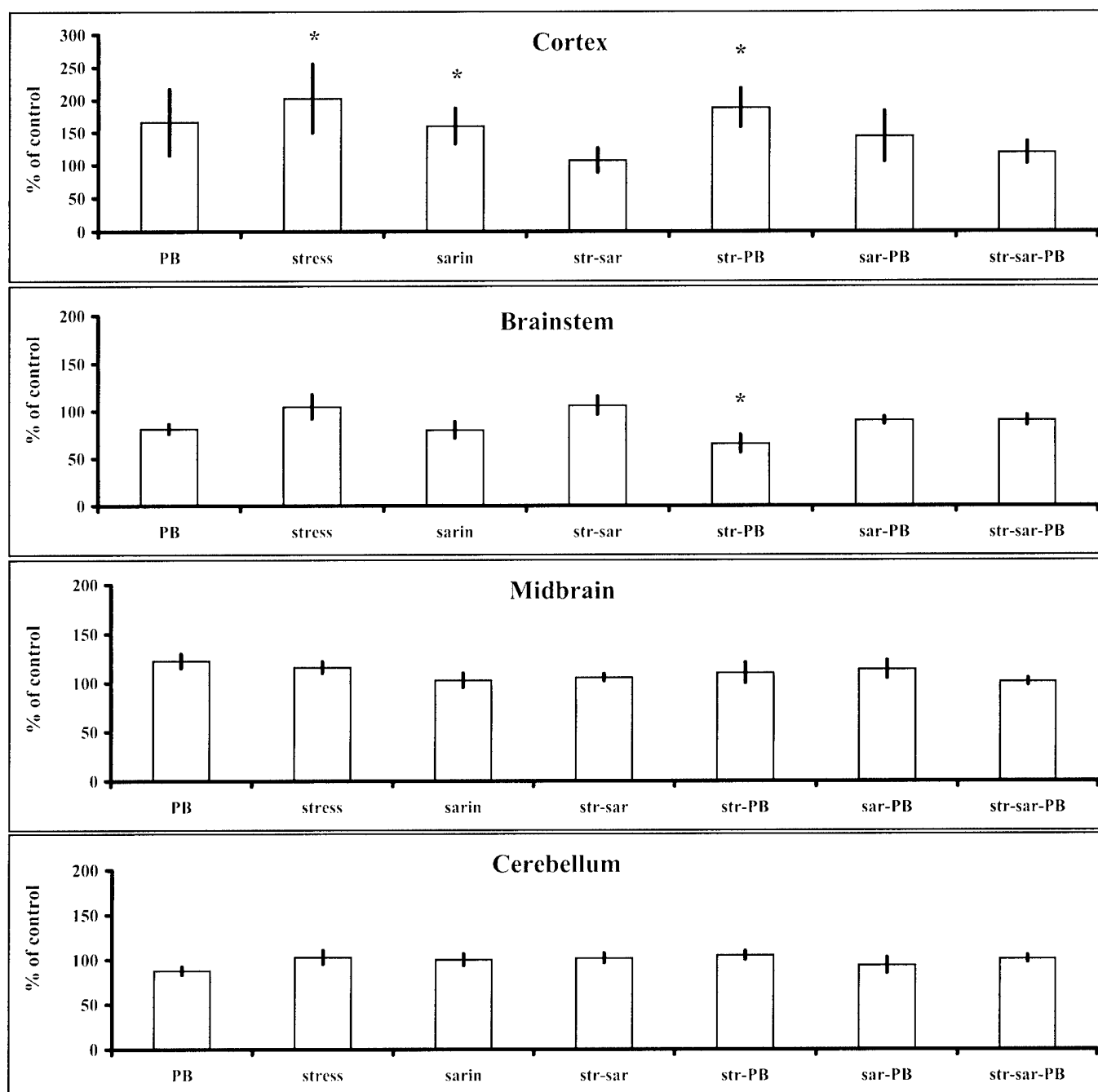


Figure 3 A : Brain regional AChE activity (PB/sarin/satress -3months)

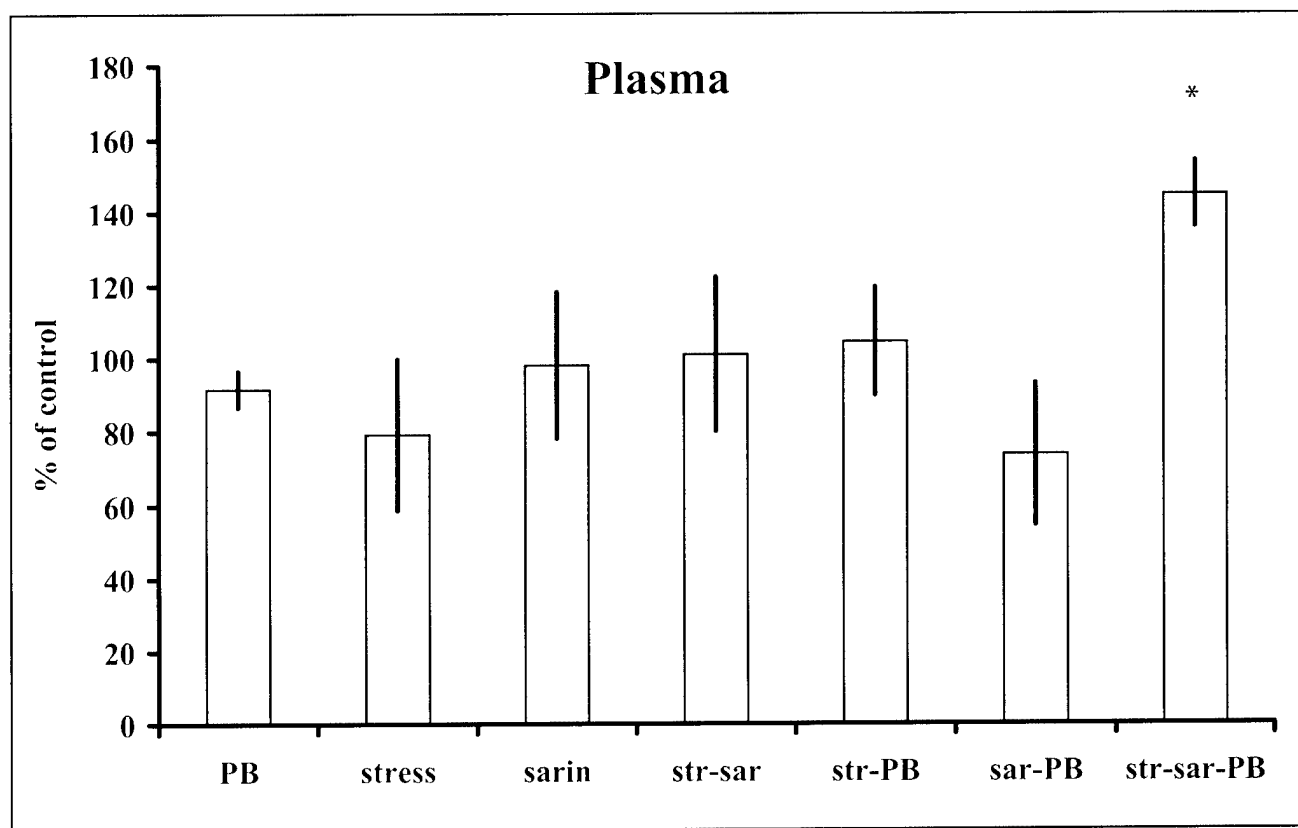


Figure 3 B : Plasma BChE activity (PB/sarin/satress -3months)

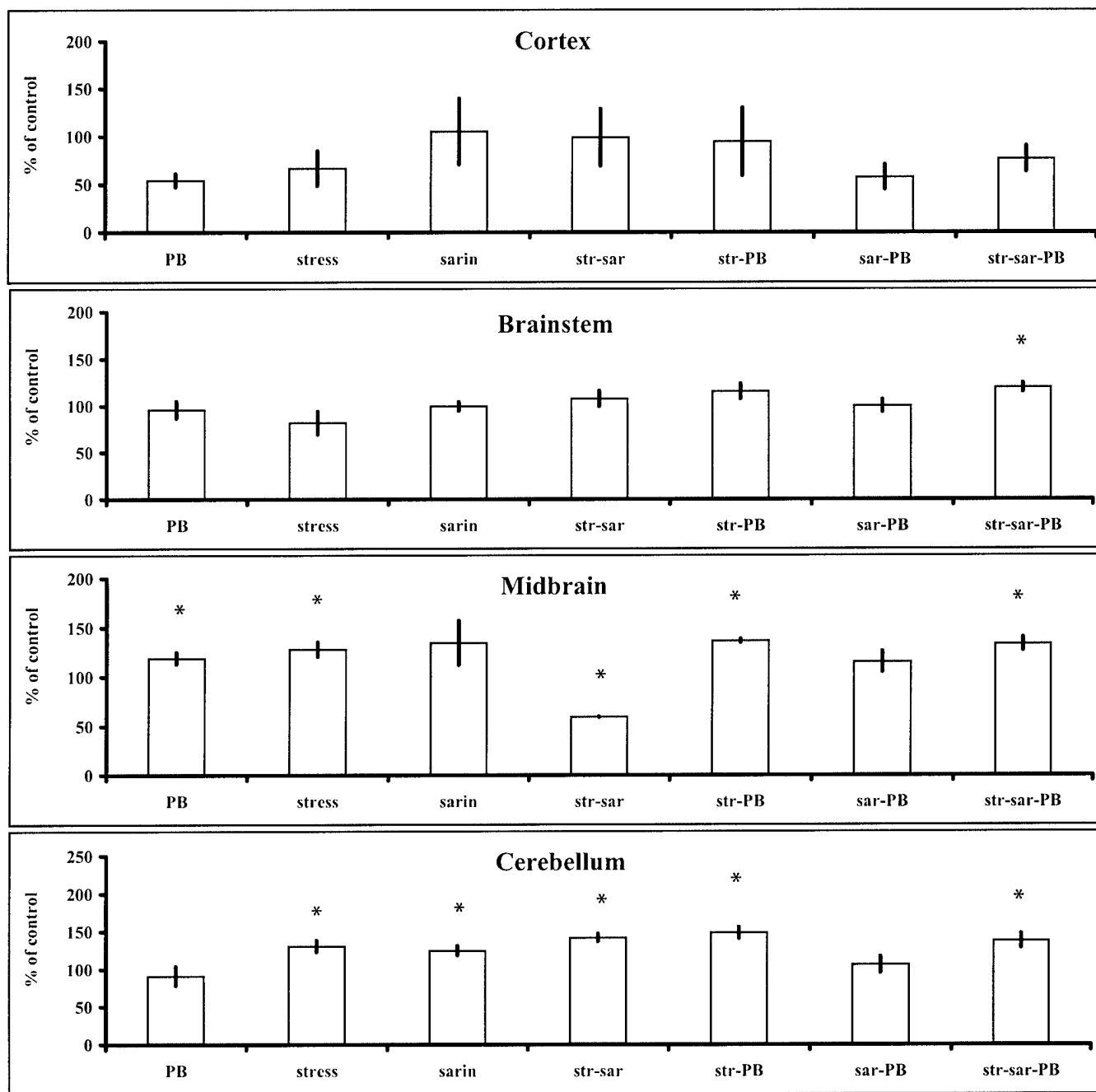


Figure 4 A : Brain regional AChE activity (PB/sarin/stress- 6 months)

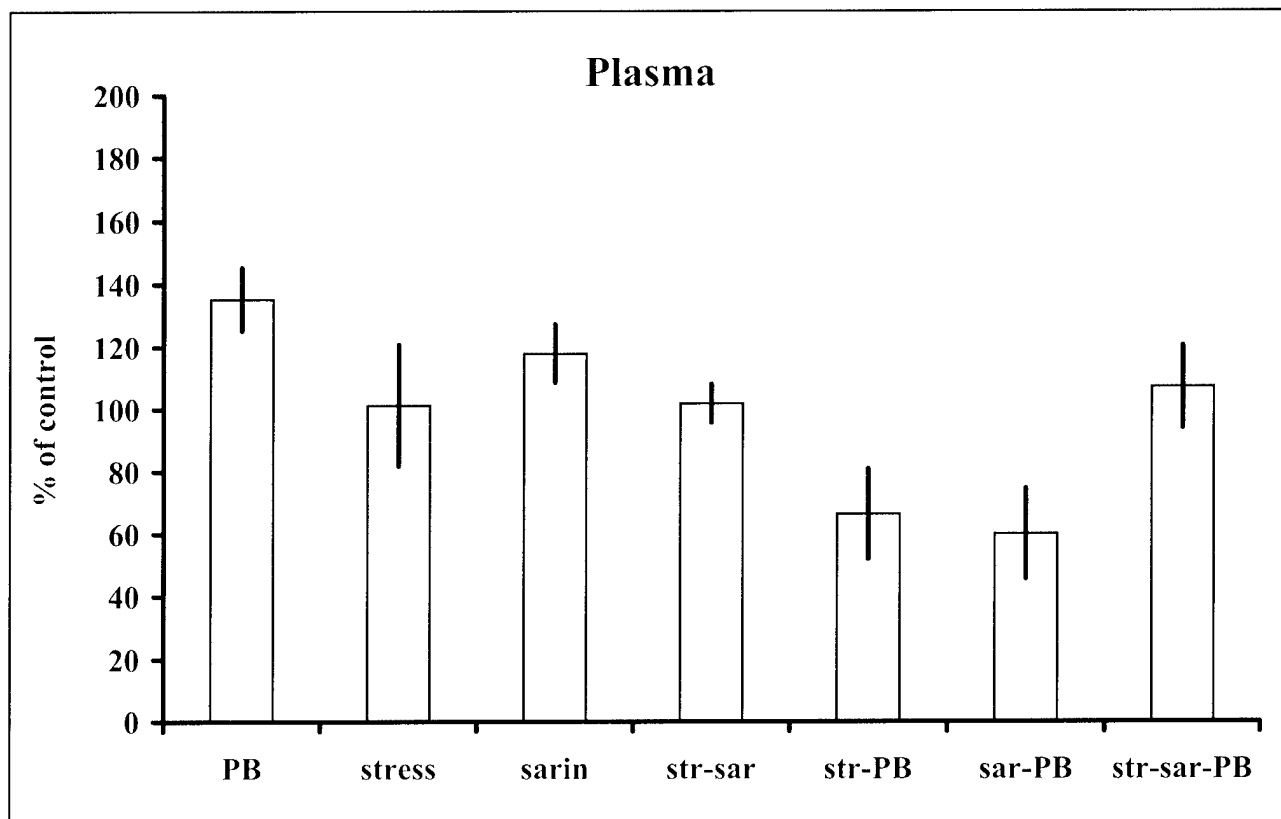


Figure 4 B : Plasma BChE activity (PB/sarin/stress - 6 months)

Key Research accomplishments:

Following are the major research accomplishments of this project:

1. Differential regulation of the cholinergic pathway in the central nervous system following acute exposure to 0.01, 0.1, 0.5 and 1xLD50 sarin:

Male Sprague Dawley rats were treated with single i.m. dose of 0.01, 0.1, 0.5, and 1xLD50 sarin (1, 10, 50, and 100 µg/kg, respectively). The animals were sacrificed after 15 hrs of the treatment. Brain regional acetylcholinesterase (AChE) and plasma butyrylcholinesterase (BChE) activity, nicotine and muscarinic receptor ligand binding in brainstem and cortex was carried out. Treatment with 1xLD50 sarin only resulted in a significant inhibition in AChE activity in all the brain regions. There was a significant increase in cortical and brainstem choline acetylcholine transferase activity at 6hrs that persisted up to 20 hrs following 1xLD50 dose. There was no significant inhibition of plasma BChE at 20 hrs at doses lower than 1xLD50 sarin. Treatment with 1xLD50 dose caused a biphasic response in cortical nicotinic and muscarinic acetylcholine receptor ligand binding. A decrease at 1 and 3hr and consistent increases at 6, 15, and 20 hrs in nAChR and m2 mAChR were observed following 1xLD50 dose (Khan et al, 2000).

2. Subchronic dose-response study following single i.m. dose of sarin:

Subchronic neurotoxic effects following treatment with single i.m. dose of 0.01, 0.1, 0.5 and 1xLD50 sarin were studied after 90 days of the treatment. Blood brain barrier permeability was monitored by the uptake of [³H]hexamethonium iodide. No significant changes were observed in brain regions except that brainstem showed a significant decrease (~50% of control). Plasma BChE activity did not show any changes at this time point. AChE activity in the cortex showed a decrease (~29% of control) whereas

brainstem showed an increase (~20% of control) at 1xLD50 dose. Cortex showed a decrease in m2 muscarinic acetylcholine receptor and brainstem exhibited an increase (~45% of control) at 1xLD50 dose. Cortex also showed a biphasic response in nicotinic acetylcholine receptor ligand binding at 0.1 and 1xLD50 doses (Jones et al, 2000).

3. Sensorimotor deficit and cholinergic changes following co-exposure with PB and sarin in rats:

In these studies, we investigated the effects of PB and sarin alone, and in combination on sensorimotor behavior and the central cholinergic system of rats. Animals were treated with 15 daily oral dose of 1.3 mg/kg PB followed by a single i.m. dose of 0.5, 0.75, 0.9 or 1xLD50 of sarin. Other group of rats were treated with PB or sarin alone. The animals were evaluated for postural reflexes, limb placing, orienting to vibrissae touch, incline plane performance, beam-walk time and forepaw grip time 7 and 15 days following treatment with sarin. Treatment with either PB or sarin alone resulted in significant sensorimotor impairments. Co-exposure to sarin and PB resulted in significant in sensorimotor deficits that continued to worsen with time. At higher doses of sarin, co-exposure to PB resulted in an additive detrimental effect on sensorimotor function. Cortical acetylcholinesterase (AChE) activity remained inhibited in the animals treated with each dose of sarin alone and in combination with PB. Cortex and brainstem muscarinic acetylcholine receptor (m2 mAChR) ligand binding showed significant increases (~120-130% of control) following co-exposure to PB and sarin at higher doses. Acute exposure to either 0.1x or 1x LD50 sarin (single i.m. injection) for 3 hrs with or without 15 days of oral pretreatment with PB (1.3 mg/kg/day) showed significant inhibition in plasma BChE activity following treatment with 1xLD50 sarin (~30% of

control). Pretreatment with PB offered slight protection. AChE activities in brain regions exhibited significant inhibition following treatment with 1xLD50 sarin and pretreatment with PB afforded a slight protection. However, the AChE activities in the brain regions of the animals treated with 1xLD50 sarin that were pretreated with PB, remained significantly inhibited as compared from the controls. These results show that treatment with sarin or PB resulted in sensorimotor impairments, and that co-exposure to high doses of sarin with PB caused an additive detrimental effect on sensorimotor performance (Abou-Donia et al, 2002).

4. Neuropathological changes in the central nervous system (CNS) of rats following exposure with 0.01, 0.1, 0.5 and 1xLD50 sarin.

In these studies, two groups of male Sprague-Dawley rats were exposed to single i.m. dose of sarin (0.01, 0.1, 0.5, and 1x LD50 equivalent to 1, 10, 50, and 100µg/kg). Following twenty four hrs after sarin treatment, there was a significant inhibition (~58 and 70% of control, $p < 0.04$) in plasma BChE in the animals treated with 0.5 and 1x LD 50 sarin. Cortex, brainstem, midbrain and cerebellum AChE activity was significantly inhibited (~54%-69% of control, $p < 0.02$) only at 1xLD 50 dose. A differential response in m2 muscarinic acetylcholine receptor ligand binding in the cortex and brainstem was observed. Sarin treatment resulted in significant impairment in the BBB permeability. MAP-2 immunostaining suggested neuronal dendritic loss in several brain regions (Abdel-Rahman et al, 2002).

5. GFAP and vimentin mRNA expression in the CNS following single i.m. treatment with 0.5xLD50 (50µg/kg) sarin.

The expression profile of mRNA for GFAP and vimentin in various brain regions of rat was studied in male Sprague Dawley rats. Following treatment with single i.m. dose of sarin (0.5xLD 50, 50g/kg) for 1, 3 hrs, and 1, 3, and 7 days there was a significant induction of mRNA for GFAP and vimentin in cortex, brainstem, midbrain, cerebellum and spinal cord. The expression of these genes showed a clear spatio-temporal difference in each region. Both the GFAP and vimentin were induced at early time point in all the regions except brainstem where moderate to high levels were observed at 1 and 3 days, respectively following treatment with sarin. Expression of GFAP and vimentin remained at high levels in brainstem, cerebellum and midbrain at all the time periods, whereas expression pattern in the cortex and spinal cord returned to normal levels by 7 days post treatment. These data indicate that the development of long-term sarin-induced neuropathological abnormalities may involve changes in the gene expression pattern of the components of astroglial cytoskeleton (Damodaran et al, 2002).

6. Altered expression of α tubulin mRNA expression in the CNS following single i.m. treatment with 0.5xLD50 (50 μ g/kg) sarin.

The expression profile of mRNA for α tubulin in various brain regions of rat was studied in male Sprague Dawley rats. Following treatment with single i.m. dose of sarin (0.5xLD 50, 50g/kg) for 1, 3 hrs, and 1, 3, and 7 days there was a significant induction of mRNA for α tubulin in cortex, brainstem, midbrain, cerebellum and spinal cord. The expression showed a spatio-temporal difference in each region, similar to the expression pattern of other cytoskeletal proteins such as GFAP and vimentin. These data indicate that the development of long-term sarin-induced neuropathological abnormalities may involve

changes in the gene expression pattern of the components of astroglial cytoskeleton (Damodaran et al, 2002).

7. Combined exposure to sarin and pyridostigmine bromide causes increased levels of urinary 3-nitrotyrosine and 8-hydroxy-2'-deoxyguanosine, biomarkers of oxidative stress:

In the present study, the possibility of the generation of oxidative stress following exposure to sarin and PB, alone and in combination was studied in male Sprague Dawley rats. Urinary levels of 3-nitrotyrosine and 8-hydroxy-2'-deoxyguanosine, as markers of oxidative stress, were determined by high performance liquid chromatography. Treatment of the rats with a combination of a single oral dose of PB (13 mg/kg, gavage) and a single i.m. dose of sarin (80µg/kg) resulted in a significantly increased levels of urinary levels of 3-nitrotyrosine and 8-hydroxy-2'-deoxyguanosine at 48 hrs after the treatment and stayed elevated upto 96 hrs following the treatment. Treatment with either PB or sarin alone did not cause an increased excretion of these biomarkers of oxidative stress in the urine.

8. Effect of stress on sub-clinical sarin exposure.

In these studies, the rats were stressed for 15 days by placing them in a plastic restrainer for 5 minutes. On the last day, following the treatment with stress, the animals were treated with single i.m. dose of 0.5xLD50 sarin. The animals were evaluated for neurobehavioral performance at 3 and 6 months following sarin treatment. With the exception of beam-walk scores, each treatment resulted in sensorimotor impairments as reflected on each test and at both the time points. However, there was no additive effect when the rats were exposed to sarin and stress in combination. For beam-walk time,

incline plane and forepaw grip time, there were significant effects following each treatment on 3 and 6 months time points as compared to controls.

Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) were determined at 24 hrs, 3 months and 6 months after treatment with 0.5xLD50 sarin. Treatment with sarin alone did not cause any significant in brain regional or plasma cholinesterase activity except that in the cortex, where there was a nonsignificant decrease (~76% of control) at 24 hrs after the sarin treatment. Treatment with stress alone, on the other hand, caused a significant increase in brainstem (~138% of control) and midbrain (~127% of control) AChE activity. Following 3 months after sarin treatment, there was a significant increase in cortex AChE activity (~159% of control) following treatment with sarin alone. A significant increase in cortex AChE activity (~202% of control) was observed in the rats treated with stress alone at 3 months. Following 6 months after sarin treatment, there was a significant increase (~124% of control) in cerebellum AChE activity in the rats treated with sarin alone. Stress exposure alone resulted in a significant increase in midbrain and cerebellum AChE activity (~128% of control). Treatment with sarin and stress in combination caused a significant decrease in midbrain AChE activity (~59% of control).

9. Effect of heat on sub-clinical sarin exposure.

In these studies, the rats were subjected to heat treatment by placing them in a biological incubator for 4 hrs. Following the treatment with heat, the animals were treated with single i.m. dose of 0.5xLD50 sarin. The animals were evaluated for neurobehavioral performance at 3 and 6 months following sarin treatment. Each treatment resulted in sensorimotor impairments in each test, and at 3 and 6 months following sarin treatment.

However, there was no additive effect when the rats were exposed to sarin and heat in combination. For incline plane, the rats treated with heat and sarin did not any further deterioration. In fact, at 3 month time point, the rats exposed to heat and sarin showed improvement in incline plane response. All the parameters showed a worsening response over 6 month period following treatment with sarin.

Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) were determined after 24 hrs, 3 months and 6 months, following treatment with single i.m. dose of 0.5xLD₅₀ sarin. At 24 hrs after sarin treatment, there no significant change in brain regional or plasma cholinesterase activity except that in the cortex, where there was a nonsignificant decrease (~76% of control) at 24 hrs after the sarin treatment. Treatment with heat alone, on the other hand, caused a significant increase in brainstem (~138% of control) and midbrain (~127% of control) AChE activity. Treatment with the combination of sarin and heat did not result in any significant change. At 3 month following sarin treatment, here was a significant increase in brainstem and cerebellum AChE activity (~120% of control) following treatment with heat alone and treatment with heat and sarin in combination (~ 122 & 130% of control). At 6 months after the treatment sartin, there was a significant increase (~115-147% of control) in brainstem, midbrain and cerebellum AChE activity in the rats treated with heat alone. Sarin exposure alone resulted in a significant increase in midbrain AChE activity (~144% of control). Treatment with heat and sarin in combination caused a significant increase in midbrain AChE and plasma BChE activity (~148% and 217% of control, respectively). These results suggest that exposure to heat, alone or in combination could

cause long-term increases in the cholinesterase activity in midbrain that may result in neurological consequences.

10. Effect of prophylactic treatment with pyridostigmine bromide (PB) on sub-clinical sarin exposure.

In these studies, the rats were treated with 1.3 mg/kg PB (oral by gavage, daily for 15 days). On the last day, following treatment with PB, the animals were treated with single i.m. dose of 0.5xLD50 sarin. The animals were evaluated for neurobehavioral performance at 3 and 6 months following sarin treatment. Beam-walk score showed no significant impairments at 3 months following either treatment PB or sarin alone. At 6 months, there was significant impairment in the animals treated with PB alone. For beam-walk time, incline plane and fore-paw grip time, there was significant effects of treatment groups at both the time points. However, performance over time was variable. However, there was no dramatic additive effect when the rats were exposed to sarin and PB in combination.

Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) were determined at 24 hrs, 3 months and 6 months after treatment with single i.m. dose of 0.5xLD50 sarin. At 24 hrs of the treatment with sarin alone did not cause any significant change in brain regional or plasma cholinesterase activity. At 3 months, there was a significant increase in cortex AChE activity (~159% of control) following treatment with sarin alone. At 6 months after sarin treatment, there was a significant increase in midbrain AChE activity (~119% of control) in the animals treated with PB alone and an increase in cerebellum AChE activity in the animals treated with sarin alone. Treatment with a combination of PB and sarin did not result in any

significant change in brain regional AChE activity at 6 month time point. These results suggest that exposure to a combination of PB and sarin does not cause long-term changes in the cholinesterase activity in brain regions.

11. Effect of treatment with PB after sub-clinical sarin exposure.

In these studies, the rats were first treated with single i.m. dose of 0.5xLD₅₀ sarin following which, they were treated with 1.3 mg/kg PB (oral by gavage, daily for 15 days). The animals were evaluated for neurobehavioral performance at 3 and 6 months following the last treatment with PB. At 3 months, beam-walk score showed a significant impairments following either treatment with sarin alone and PB in combination with sarin. At 6 months, there was significant impairment in the animals treated with PB alone or PB in combination with sarin. For beam-walk time, animals treated with PB or PB in combination with sarin performed poorly. Incline plane and fore-paw grip time, there was significant effects of treatment groups at both the time points. However, performance over time was variable. However, there was no dramatic additive effect following exposure to a combination of sarin and PB.

Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) were determined at 24 hrs, 3 months and 6 months, following the last dose of PB. At 24 hrs after the last dose of PB, treatment with sarin alone caused a significant increase in brainstem and midbrain AChE activity (~208 and 217% of control) whereas treatment with a combination of PB and sarin caused a significant increase (~223% of control) in brainstem AChE activity. At 3 months time period, there was a significant increase in brainstem and midbrain AChE activity (~108 and 117%% of control for brainstem and ~121 and 130% of control for midbrain))

following treatment with sarin and PB alone. Brainstem also exhibited a significant increase (~116% of control) in AChE activity following combined treatment with PB and sarin. At 6 months after the treatment with the last dose of PB, there was a significant increase in brainstem AChE activity (~112 and 130% of control) in the animals treated with PB and sarin alone. Treatment with a combination of PB and sarin did not result in any significant change in brain regional AChE activity at 6 month time point. These results suggest that exposure to a combination of PB and sarin, when PB was given after exposure to sarin, does not cause long-term changes in the cholinesterase activity in brain regions.

12. Effect of stress and prophylactic treatment with PB on sub-clinical sarin exposure.

In these studies, the rats were first treated with 1.3 mg/kg PB (oral by gavage, daily for 15 days) or with stress (by placing them in a restrainer for 5 minutes, daily for 15 days) or with the combination of PB and stress, following which they were treated with single i.m. dose of 0.5xLD₅₀ sarin. The animals were evaluated for neurobehavioral performance at 3 and 6 months following the last treatment with PB. Beam-walk score was significantly impaired at 3 months in the animals treated with stress and a combination of stress with PB. Treatment with stress and PB alone and a combination of stress with either PB or sarin resulted in significant impairments at 6 month. For beam-walk time, incline plane, and forepaw grip there was a significant impairment in all the treated groups and at 3 and 6 month time periods. However, there was no additive effect when the rats were exposed to PB, sarin and stress in combination.

Acetylcholinesterase (AChE) activity in different regions and plasma butyrylcholinesterase (BChE) were determined at 24 hrs, 3 months and 6 months after treatment with single i.m. dose of 0.5xLD50 sarin. At 24 hrs, stress caused a significant increase in brainstem and midbrain AChE activity (~138 and 127% of control, respectively). Treatment with a combination of stress and PB and stress in combination with PB and sarin resulted in a significant increase in midbrain AChE activity (~125 and 118% of control). At 3 month, there was a significant increase in cortex AChE activity (~159% of control) following treatment with sarin alone (~159% of control), stress alone (~202% of control), and a combination of stress and PB (~188% of control). A significant increase in cortex AChE activity (~202% of control) and a significant decrease in AChE activity in the brainstem (~59% of control) was observed in the rats treated with stress and PB in combination. At 6 months, there was a significant increase in midbrain AChE activity in the animals treated with PB alone (~119% of control), stress alone (~128% of control) and stress and PB in combination (~136% of control) and a significant decrease in stress in combination with sarin (~56% of control). The cerebellum AChE activity showed an increase in the rats treated with sarin alone (~124% of control) or stress alone (~130% of control) or a combination of stress and sarin (~141% of control) or stress and PB (~148% of control). Exposure to a combination of sarin, PB and stress resulted in a significant increase in AChE activity in brainstem (120% of control), midbrain (~133% of control) and cerebellum (~137% of control).

Reportable Outcome

The major objective of this proposal is to investigate the consequences of long-term, low-level exposure to sarin, alone, and in combination with pyridostigmine bromide (PB) and stress on the development of chronic and neurologic deficits. Sarin, an organophosphorus ester, is designed to specifically inhibit acetylcholinesterase (AChE) in the central and peripheral nervous systems. The function of AChE is to terminate nerve impulse transmission by hydrolyzing the neurotransmitter acetylcholine (ACh). Accumulation of ACh at the neuromuscular junctions and other cholinergic synapses causes overstimulation and disruption of the cholinergic system. The major reportable outcome of our studies are:

1. An important finding from our studies is that brain AChE and plasma BChE inhibition might be used as a biomarker for exposure, not for sarin-induced neurologic deficits. Rats treated with toxic doses of sarin showed inhibition of brain and plasma cholinesterases, that subsequently recovered. On the other hand, these animals exhibited sensorimotor deficit that persisted throughout the six-month observation period.
2. Another significant result from our studies is that a brief insult from a single lethal or sub-lethal dose of sarin, can generate a series of events to result in a diffuse neuronal cell death. Neuronal degeneration was exhibited in the cerebral cortex, the hippocampus, and Purkinje cell neurons in the cerebellum. These lesions were accompanied by increased blood brain barrier permeability and transient inhibition of AChE. These findings suggest additional mechanisms for sarin-induced neurotoxicity in the central nervous system.
3. Stress, caused by immobilization or heat also exacerbated sarin-induced neurotoxicity.
4. The results show that prophylactic treatment with PB offered some protection of peripheral AChE against sarin inhibition. On the other hand, PB exacerbated the toxicity of high doses

of sarin. Furthermore, the results showed that treatment with either sarin or PB alone resulted in sensorimotor impairments that persisted throughout the six-month observation period.

Although the mechanisms by which sarin induces neuronal lesions are not known, there is evidence that oxidative stress may play a major role in this process. First, brain regions that exhibited neuroathological lesions, i.e., motor cortex, somatosensory cortex, CA1 and CA3 subfields of the hippocampus, and Purkinje cells of the cerebellum, are all known to be vulnerable to the oxidative stress. Second, exposure to sarin in combination with PB, produces free radical species leading to increased 8-hydroxy-2'-deoxyguanosine, and 3-nitrotyrosine, resulting from excessive generation of reactive oxygen species (ROS) or by decreased repair efficiency of the oxidative damage. These results are consistent with the earlier finding that PB produced a dose-related apoptosis in the rat brain. The mechanism of PB-induced apoptosis investigated in rat cerebellar granule cells demonstrated that ROS generated by excessive activation of muscarinic and NMDA receptors initiated apoptotic events.

The cytoskeletal abnormalities (in addition to neuronal cell death) in both the cerebral cortex and the hippocampus, suggest injury to these neurons. Previous studies also indicate that the reduction of MAP-2 expression in neurons following excitotoxic brain damage is likely related to alterations in the increased concentration of intracellular calcium. This is because increased intracellular calcium concentration can mediate alterations of MAP-2 expression both by influencing the activity of kinases and phosphatases, all of which can rapidly proteolyze MAP-2 and dramatically reduce the amount of MAP-2 within neurons. Also, an increased intracellular calcium concentration can lead to an excessive degradation of MAP-2 by calpain and result in neuronal dysfunction. Both degeneration of neurons and reductions in the expression of MAP-2

in surviving neurons of layers III and V of the motor cerebral cortex and the hippocampal formation are of significant importance. This is because layer III and V neurons in the cortex are the source of axons of the corticospinal tract, which is the largest descending fiber tract (or motor pathway) from the brain controlling movements of different contralateral muscle groups. Thus, significant death of layer V neurons of the motor cortex following exposure to sarin could lead to weakness and loss of strength as well as problems with gait and coordination of movement. Further, disruption of hippocampal circuitry due to degeneration of neurons in different subfields can lead to learning and memory deficits. The above changes likely explain some of the symptoms such as loss of memory, muscle weakness, and alterations in learning ability observed in Gulf War Veterans.

Conclusions:

The goal of the current project is to study the long-term neurotoxic and neuropathological effects associated with exposure to low-dose sarin alone, and in combination with pyridostigmine bromide (PB) and stress. Additionally, we also evaluated the neurotoxicity of sarin in combination with environmental conditions such as heat stress. Various experimental paradigms were considered to create conditions similar to those present during the PGW. These results will help in understanding the molecular mechanisms of neuropathological effects resulting as a consequence of co-exposure to sarin in combination with a variety of exposure conditions.

1. Acute exposure to 0.01, 0.1, 0.5 and 1xLD50 sarin causes differential regulation of the cholinergic pathway in the central nervous system. Treatment with 1xLD50 sarin only resulted in a significant inhibition in AChE activity in all the brain regions 24 hrs after exposure. At lower doses than 1xLD50, no significant change in brain regional or plasma cholinesterase activity is observed. nAChR and m2 mAChR ligand bindings showed a decrease at 1 and 3hr and an increases at 6, 15, and 20 hrs following exposure to 1xLD50 dose.

2. Subchronic neurotoxic effects following treatment with single i.m. dose of 0.01, 0.1, 0.5 and 1xLD50 sarin were studied after 90 days of the treatment showed a decrease in AChE activity in the cortex (~29% of control), whereas brainstem showed an increase (~20% of control) at 1xLD50 sarin. Cortex showed a decrease in m2 muscarinic acetylcholine receptor and brainstem exhibited an increase (~45% of control) at 1xLD50 dose. Cortex also showed a biphasic response in nicotinic acetylcholine receptor ligand binding at 0.1 and 1xLD50 doses.

3. Treatment with either PB or sarin alone resulted in significant sensorimotor impairments.
4. Co-exposure to sarin and PB resulted in significant in sensorimotor deficits that continued to worsen with time.
5. At higher doses of sarin, co-exposure to PB resulted in an additive detrimental effect on sensorimotor function.
6. Treatment with PB alone, and a combination of PB and sarin, in increased plasma BChE activity.
7. Cortical acetylcholinesterase (AChE) activity remained inhibited in the animals treated with each dose of sarin alone and in combination with PB. PB treatment alone resulted in an increase in AChE activity in all the brain regions.
8. Muscarinic acetylcholine receptor (m_2 mAChR) ligand binding in cortex and brainstem showed significant increases (~120-130% of control) following co-exposure to PB and sarin at higher doses.
9. Following acute exposure with sarin alone or in combination with 15 days pretreatment with PB, a significant inhibition in plasma BChE activity occurred following treatment with 1xLD50 sarin (~30% of control). Pretreatment with PB offered slight protection, but the plasma BChE activity still remained inhibited (~70% of control) following treatment with 1xLD50 sarin.
10. AChE activity in cortex, brainstem, midbrain and cerebellum exhibited significant inhibition following treatment with 1xLD50 sarin and pretreatment with PB afforded a slight protection. However, the AChE activities in the brain regions of the animals

treated with 1xLD50 sarin that were pretreated with PB, remained significantly inhibited as compared from the controls.

11. Cortex m2 mAChR ligand binding densities were increased significantly following treatment with 0.1xLD50 sarin and PB alone.

12. A significant inhibition (~58 and 70% of control, $p < 0.04$) in plasma BChE was observed following treatment 0.5 and 1x LD 50 sarin.

13. Cortex, brainstem, midbrain and cerebellum AChE activity was significantly inhibited (~54%-69% of control, $p < 0.02$) only at 1xLD 50 dose.

14. Significantly higher immunostaining was observed with EBA antibodies, suggesting BBB permeability changes following treatment with sarin.

15. Immunohistochemical staining with GFAP and MAP-2 revealed mild glial reactivity and neuronal dendritic loss in several brain regions.

16. Treatment with single i.m. dose (0.5xLD 50) of sarin resulted in mRNA induction of GFAP and vimentin in all the brain regions examined and spinal cord.

17. The expression of these genes showed a clear spatio-temporal difference in each region.

18. Both the GFAP and vimentin were induced at early time point in all the regions except brainstem where moderate to high levels were observed at 1 and 3 days, respectively following treatment with sarin.

19. Expression of GFAP and vimentin remained at high levels in brainstem, cerebellum and midbrain at all the time periods, whereas expression pattern in the cortex and spinal cord returned to normal levels by 7 days post treatment.

20. Cortex and spinal cord showed the highest levels of expression of GFAP (~318% and 368%, respectively).
21. Expression of vimentin mRNA reached to highest levels only in cortex (~284%).
22. The changes observed in the expression pattern of GFAP and vimentin could be due to astroglial cell proliferation resulting as a consequence of astrocyte loss, astrocytic dysfunction and changes in the functional status of neuronal cells.
23. Treatment with subclinical dose of sarin (0.5xLD₅₀, i.m., single dose) resulted in sensoromotor deficit at 3 and 6 months following treatment.
24. A combination of stress and heat also caused significant impairments in the neurobehavioral performance at 3 and 6 months.
25. The long-term effects of sarin on AChE activity showed no effects in most of the brain regions, suggesting that AChE activity measurements may not be a reliable marker for long-term toxic effects following sarin exposure.

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Appendix 1: Manuscripts published

1. Khan, W.A., Dechkovskaia, A.M., Herrick, E.A., Jones, K.H., and Abou-Donia, M.B. (2000). Acute sarin exposure causes differential regulation of choline acetyltransferase, acetylcholinesterase, and acetylcholine receptors in the central nervous system of the rat. *Toxicol. Sci.* **57**: 112-120.
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Acute Sarin Exposure Causes Differential Regulation of Choline Acetyltransferase, Acetylcholinesterase, and Acetylcholine Receptors in the Central Nervous System of the Rat

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Acute neurotoxic effects of sarin (*O*-isopropylmethylphosphonofluoridate) in male Sprague-Dawley rats were studied. The animals were treated with intramuscular (im) injections of either $1 \times LD_{50}$ (100 μ g/kg), and sacrificed at 0.5, 1, 3, 6, 15, or 20 h after treatment, or with im injections of either 0.01, 0.1, 0.5, or $1 \times LD_{50}$ and sacrificed 15 h after treatment. Plasma butyrylcholinesterase (BChE) and brain regional acetylcholinesterase (AChE) were inhibited (45–55%) by 30 min after the LD_{50} dose. BChE in the plasma and AChE in cortex, brainstem, midbrain, and cerebellum remained inhibited for up to 20 h following a single LD_{50} treatment. No inhibition in plasma BChE activity was observed 20 h after treatment with doses lower than the LD_{50} dose. Midbrain and brainstem seem to be most responsive to sarin treatment at lower doses, as these regions exhibited inhibition ($\sim 49\%$ and 10% , respectively) in AChE activity following $0.1 \times LD_{50}$ treatment, after 20 h. Choline acetyltransferase (ChAT) activity was increased in cortex, brainstem, and midbrain 6 h after LD_{50} treatment, and the elevated enzyme activity persisted up to 20 h after treatment. Cortex ChAT activity was significantly increased following a $0.1 \times LD_{50}$ dose, whereas brainstem and midbrain did not show any effect at lower doses. Treatment with an LD_{50} dose caused a biphasic response in cortical nicotinic acetylcholine receptor (nAChR) and muscarinic acetylcholine receptor (m2-mAChR) ligand binding, using [³H]cytisine and [³H]AFDX-384 as ligands for nAChR and mAChR, respectively. Decreases at 1 and 3 h and consistent increases at 6, 15, and 20 h in nAChR and m2-mAChR were observed following a single LD_{50} dose. The increase in nAChR ligand binding densities was much more pronounced than in mAChR. These results suggest that a single exposure of sarin, ranging from 0.1 to $1 \times LD_{50}$, modulates the cholinergic pathways differently and thereby causes dysregulation in excitatory neurotransmission.

Key Words: sarin; choline acetyltransferase; acetylcholinesterase; muscarinic acetylcholine receptor; nicotinic acetylcholine receptor; neurotoxicity; Gulf War.

War, several hundred thousand American soldiers were presumably exposed to a combination of neurotoxic chemicals, and possibly to sarin (Institute of Medicine, 1995). Some of these veterans complained of symptoms that involved defects in, or abnormal regulation of, the central and peripheral cholinergic nervous system (Persian Gulf Veterans Coordination Committee, 1995).

The neurotoxicity of sarin has been evaluated in different rodent and mammalian species, and the acute toxic effects are supposed to be mediated by inhibition of acetylcholinesterase (AChE). The main clinical features associated with acute sarin intoxication are seizure, fasciculation, tremor, and hypothermia (Taylor, 1985). The appearance of these symptoms correlates with the inhibition of AChE, both in the central nervous system (CNS) and peripheral nervous system (PNS) (Gupta *et al.*, 1991). This is followed by excessive accumulation of acetylcholine, leading to hyperactivation of nicotinic and muscarinic acetylcholine receptors.

Excessive accumulation of acetylcholine leads to activation of ligand-gated ion channels, and of nicotinic acetylcholine receptors (nAChR), and muscarinic acetylcholine receptors (mAChR). These receptors activate diverse kinds of cellular responses by distinct signaling mechanisms (Wess, 1996). Indeed, previous studies from our laboratory and others have shown that organophosphate compounds cause differential regulation of nAChR and mAChR (Huff *et al.*, 1994; Katz *et al.*, 1997). *In vitro* studies by Bakry *et al.* (1988) suggested that sarin binds to nAChR and modulates its ligand-binding characteristics. A recent study showed a decrease in high-affinity choline uptake by the insecticide chlorpyrifos (*O*, *O*-diethyl 3,5,6-trichloropyridenyl phosphorothioate) (Liu and Pope, 1996). The levels of acetylcholine in the CNS can be regulated by different metabolic pathways, e.g., by the inhibition or activation of AChE and choline acetyltransferase and regulation of high-affinity, sodium-dependent choline transporter (Taylor and Brown, 1999). Rats exposed to soman and sarin have been found to have a decrease in high affinity choline uptake in cortex and hippocampus (Whalley and Shih, 1989). From all of these studies, it is apparent that changes in acetylcholine-related metabolism are the key regulators of CNS

Sarin, *O*-isopropylmethylphosphonofluoridate, was developed as a warfare nerve agent (Wood, 1951). During the Gulf

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toxicity induced by organophosphate compounds, including sarin.

Several studies in the past have reported consistent inhibition of AChE by sarin; however, its effects on choline acetyltransferase (ChAT) are shown to be variable, with some having no effect (Sivam *et al.*, 1984) and others with inhibition of no consequence (Kobayashi *et al.*, 1986). Yet another study by Brookes and Goldberg (1979), using cultured spinal cord cells, showed activation of ChAT by a closely related compound, diisopropylphosphorofluoridate. Because of the central role of the cholinergic system in the manifestation of toxicity by sarin, we decided to evaluate acute effects of sarin on the interplay between the cholinergic parameters, i.e., AChE, ChAT, nAChR, and mAChR, concurrently, in a single study. Our studies show that sarin caused inhibition of plasma BChE and brain region-specific AChE and persistent activation of ChAT. They also show that sarin caused increased binding of nAChR- and mAChR-specific ligands.

MATERIALS AND METHODS

Chemicals

Butyrylcholine iodide, acetylcholine iodide, acetyl CoA, and choline chloride were obtained from Sigma Chemical Co. (St. Louis, MO). Tetraphenyl boron was from Sigma-Aldrich Chemicals (St. Louis, MO). [^3H]Acetyl CoA (sp. activity 12 Ci/mmol) was purchased from ICN Chemicals (Costa Mesa, CA). [^3H]cytosine (sp. activity, 15 Ci/mmol) and [^3H]AF-DX384[2,3 Dipropylamino] (sp. activity, 100 Ci/mmol) was obtained from NEN (Boston, MA). Rabbit polyclonal antibodies to choline acetyltransferase were obtained from Chemicon International (Tencula, CA) and also as a kind gift from Dr. L. Hersh, University of Kentucky (Louisville, KY). SDS-PAGE and Western blotting apparatus were from Biorad (Richmond, CA). ECL kit was supplied by Amersham Biosciences. All other reagents were of the highest purity available commercially. Sarin (1.90 mg/ml in saline) was obtained from the U.S. Army Medical Research and Materiel Command, Fort Detrick, MD.

Animals

Male Sprague-Dawley rats (200–250g) were obtained from Zivic-Miller Laboratories (Allison Park, PA) and housed in the Duke University Medical Center vivarium on a 12-h dark-light cycle. The animals were allowed food and water *ad libitum*. All the treatments and procedures on the animals were carried out strictly according to the recommended guidelines by the Army and the Duke University Institutional Animal Care and Use Committee.

Treatment of Animals and Tissue Retrieval

Animals were treated with a single intramuscular injection of 100 $\mu\text{g/kg/ml}$ in normal saline into the thigh muscle for the LD₅₀ time-course study (Abou-Donia, *et al.*, unpublished observation). For LD₅₀ treatments, a minimum of 10 animals were used, of which 2 to 3 died within 60 min of treatment. The remaining surviving animals were sacrificed for tissue and blood collection, to carry out biochemical estimations. For dose study, sarin was diluted with normal saline to give a final concentration of 0.01, 0.1, 0.5, or 1 \times LD₅₀ to each animal. Control animals received an equal volume of vehicle. At the termination of the experiment, the animals were anesthetized with 0.2 ml of ketamine/xylocaine and blood was drawn into a heparinized syringe. Animals were dissected, and the brain was removed and washed thoroughly with ice-cold normal saline to remove blood. Brain regions (cortex, midbrain, cerebellum, and brainstem) were dissected on ice and snap frozen in liquid nitrogen.

Enzyme and Receptor Assays

Cholinesterase determination. AChE in brain regions and BChE in plasma activities were determined according to the method of Ellman *et al.* (1961), modified for assay in a Molecular Devices UV Max Kinetic Microplate Reader, as previously described (Abou-Donia, *et al.*, 1996). In brief, brain regions were weighed and 10% homogenate was prepared in 0.1 M phosphate buffer, pH 8.0, containing 0.5% Triton X-100. The homogenate was centrifuged at 5000 \times g for 10 min at 4°C. The supernatant was used as the source of the enzyme. Blood samples were centrifuged at 5000 \times g for 10 min to separate plasma. All samples were stored at -70°C until use. All tissue supernatants were diluted 1:10 with PBS containing 10 mM MgCl₂, pH 7. Twenty μl of diluted supernatant was used for each assay in a total volume of 200 μl of buffer or 0.2 mM acetylthiocholine iodide. For plasma BChE determination, the plasma was diluted 1:10 in PBS containing 10 mM MgCl₂ and assayed as described for brain regions, except that 0.2 mM butyrylthiocholine (BSCh) was used as substrate in the presence of 5×10^{-7} M AChE inhibitor, 1,5-bis-(N-allyl-N-N-dimethyl-4-ammoniumphenyl) pentane-3-one dibromide. The reaction was started by the addition of 0.1 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in PBS. The blank contained buffer in the place of substrate, and the enzyme activity was monitored by recording the absorbance at 412 nm. Protein concentration was determined by BCA method according to Smith *et al.* (1985). The enzyme activities are expressed as μmol substrate hydrolyzed/min/mg protein for brain regions and nmol substrate hydrolyzed/min/mg protein for plasma.

Determination of choline acetyltransferase. Choline acetyltransferase (ChAT) activity in brain regions was determined according to the method of Fonnum (1975). Briefly, the tissues were homogenized in 50 mM phosphate buffer, pH 7.4, containing 0.5% TritonX-100 and centrifuged at 5000 \times g for 10 min at 4°C. The supernatant was used as the source of enzyme. The assay was carried out in 50 mM phosphate buffer, pH 7.4, containing 0.2 M sodium chloride, 10 mM EDTA, 100 μM eserine, 5 mM choline chloride, 200 μM acetyl CoA (0.25 μCi [^3H]acetyl CoA), in a final volume of 200 μl , for 30 min at 37°C. The reaction was stopped by adding an equal volume of 1.5% tetraphenyl boron in 3-heptanone, vortexed thoroughly, and centrifuged at 5000 \times g for 5 min to separate the organic phase. The acetylcholine level was determined by counting the organic phase. Enzyme activity was expressed as pmol acetylcholine formed/min/mg protein.

SDS-PAGE and Western blotting of ChAT. A suitable aliquot of 5000 \times g supernatant containing 25 μg of protein was denatured with sample buffer. Proteins were separated and transferred to PVDF membranes as described by Khan *et al.* (1994). The membranes were incubated with 5% non-fat dry milk containing 0.5% Surfactin in Tris-buffered saline, pH 7.4, for 1 h at room temperature to block the nonspecific sites. Membranes were incubated with the primary antibody overnight at 4°C at 1:1000 dilution. The membranes were washed with 5% non-fat dry milk containing Surfactin \times 3 for 15 min each, following which the incubation with secondary antibody conjugated to horseradish peroxidase was carried out for 1 h at room temperature. After extensive washing, the reaction was developed by chemiluminescence using an ECL kit supplied by Amersham Biosciences.

Nicotinic acetylcholine receptor (nAChR) binding assay. [^3H]Cytosine was used as the specific ligand for binding studies with nAChR according to the method described by Slotkin *et al.* (1999). The tissue was homogenized by polytron in 50 mM Tris-HCl, pH 7.4, containing 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, and 2.5 mM MgCl₂. The membranes were sedimented by centrifuging at 40,000 \times g for 10 min. The resulting membrane pellet was resuspended in the same buffer, using Teflon pestle glass homogenizer in a volume sufficient to give 1.5 to 2.0 mg/ml protein. An aliquot of membrane preparation containing \sim 200 μg protein was used to carry out the incubation with 1 nM [^3H]cytosine at 4°C for 75 min. Nonspecific binding was carried out in the presence of 1 μM nicotine ditartrate. The labeled membranes were trapped on membrane filters using a rapid vacuum filtration system, and the results are expressed as specific binding (dpm)/mg protein.

Muscarinic acetylcholine receptor (mAChR) binding assay. For the assay of mAChR, the tissue was homogenized in 10 mM phosphate buffer, pH 7.4, and centrifuged at $40,000 \times g$ for 10 min, and the membranes were suspended in the same buffer at the protein concentration of 1.5–2.5 mg/ml as described by Huff *et al.* (1994). Muscarinic receptor in the CNS comprises a family of 5 distinct members (m1–m5). We carried out ligand-binding studies with m2-mAChR, because of its central role in memory and learning, and our previous studies (Huff *et al.*, 1994) have shown that m2-mAChR is selectively regulated by organophosphate. The m2-mAChR binding was carried out by using m2-selective ligand, [3H] AFDX 384 at room temperature for 60 min. Nonspecific binding was carried out in the presence of 2.22 μM atropine. Ligand-bound membranes were trapped on glass filters presoaked with 0.1% polyethyleneimine using rapid vacuum filtration as described for the nAChR assay. The results are expressed as specific binding (dpm)/mg protein.

Statistical analysis. The data were analyzed by Student's *t*-test for statistical significance. The graphs were generated on Excel graphics for Macintosh and are presented as mean \pm SE.

RESULTS

Clinical Signs

All animals were observed for the development of clinical signs of toxicity. Animals treated with sarin at $1 \times LD_{50}$ showed severe tremors, seizures, and salivation within 5–15 min of treatment and these signs continued with increasing severity for up to 30 min. Death occurred in some animals that had also exhibited convulsion, possibly because of respiratory paralysis. Animals treated with $0.5 \times LD_{50}$ developed tremors by 15–30 min after treatment. Treatment with lower doses of sarin did not result in any observable signs of toxicity.

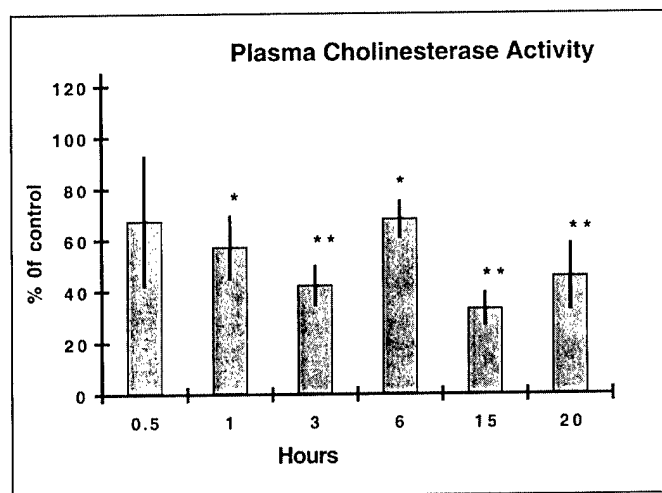


FIG. 1. Effect of sarin on plasma cholinesterase activity, time course. Animals were treated with single doses of sarin $1 \times LD_{50}$ (100 $\mu g/kg$, im). Animals were sacrificed at the indicated time periods and blood was collected in heparinized syringes. Plasma was separated and the enzyme activity was determined using butyrylthiocholine as substrate, as described in Materials and Methods. Data are presented as mean \pm SE (% of control); * $p < 0.01$ (for 6 h, $p < 0.03$), ** $p < 0.001$.

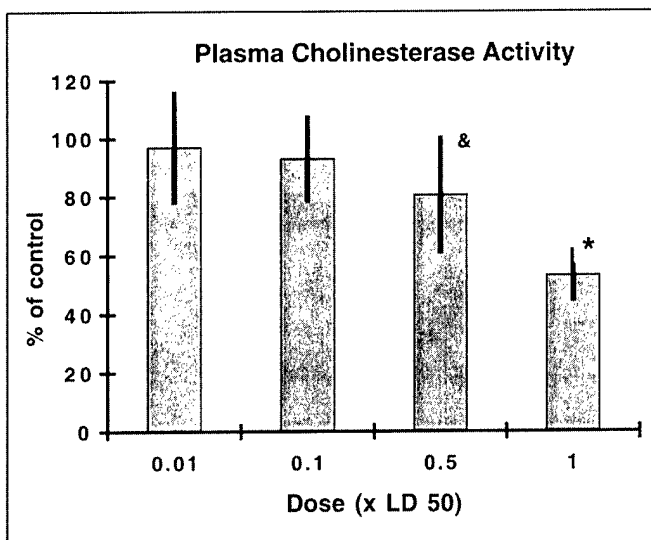


FIG. 2. Effect of sarin on plasma cholinesterase activity, dose effect. Animals were treated with im injections of 0.01, 0.1, 0.5, or $1 \times LD_{50}$ of sarin in saline and sacrificed after 15 h. Data are presented as mean \pm SE (% of control). Symbol "&" indicates not significant; * $p < 0.04$.

Effect of Sarin on Plasma Cholinesterase Activity

Initially we carried out a time course study to evaluate the inhibitory potential of sarin after a single LD_{50} dose. Sarin treatment at $1 \times LD_{50}$ dose resulted in $\sim 45\%$ decrease in plasma butyrylcholinesterase activity 30 min after exposure and continued to decrease up to 55–65% by 3 and 15 h (Fig. 1). The maximum inhibitory effect persisted up to 15 h after treatment.

Because exposure to sarin could be to doses lower than the LD_{50} , we wanted to examine the effects of sarin on plasma BChE at 0.01, 0.1, 0.5, and $1 \times LD_{50}$; we sacrificed the animals 15 h after treatment. The data in Figure 2 show that plasma cholinesterase activity was inhibited significantly only at the $1 \times LD_{50}$ dose 15 h after treatment ($p < 0.04$). However, it is likely that a smaller dose may have some inhibitory effect at earlier time periods.

Effect of Sarin on Brain Regional AChE

We studied the effect of sarin on AChE in different regions of brain after a single LD_{50} dose. The data presented in Figure 3 show the inhibition pattern of AChE in cortex, brainstem, midbrain, and cerebellum. Cortex and midbrain registered a significant decrease in AChE activity 30 min after treatment. Brainstem, midbrain, cortex, and cerebellum all exhibited statistically significant inhibition 3 h post-exposure. Only 13, 20, 16, and 20% activity remained in cortex, brain stem, midbrain, and cerebellum, respectively, after 3 h, and this inhibitory pattern was maintained for up to 15 h, after which there was some recovery in the enzyme activity.

Data on the effect of different doses of sarin, 15 h after

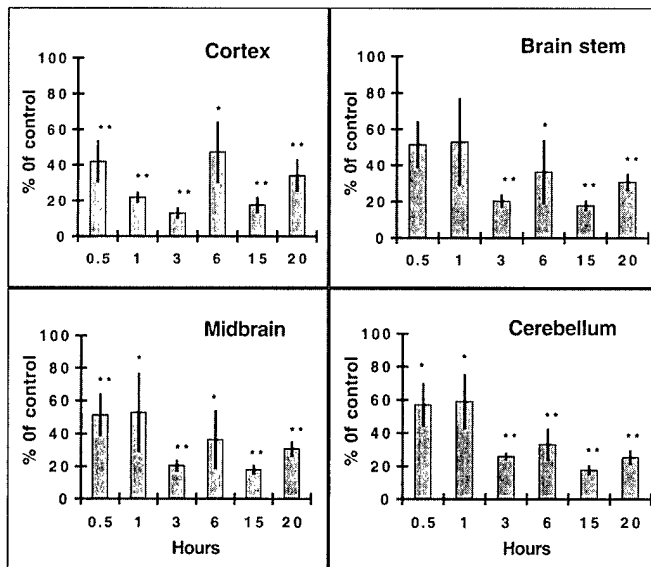


FIG. 3. Effect of sarin on brain regional AChE activity, time course. Animals were treated with single doses of sarin $1 \times LD_{50}$ ($100 \mu\text{g/kg}$, im) and brain regions were dissected at the indicated time period. The enzyme activity was determined using acetylthiocholine as substrate, as described in Materials and Methods. Data are presented as mean \pm SE (% of control); * $p < 0.01$, ** $p < 0.001$.

dosing, on brain regional AChE are presented in Figure 4. Cortex and brain stem AChE activity registered a decrease only after 0.5 and $1 \times LD_{50}$. This inhibition was statistically significant only at the $1 \times LD_{50}$ dose (~ 11 and 34% activity for cortex and brainstem, respectively, remaining after 15 h). Mid-

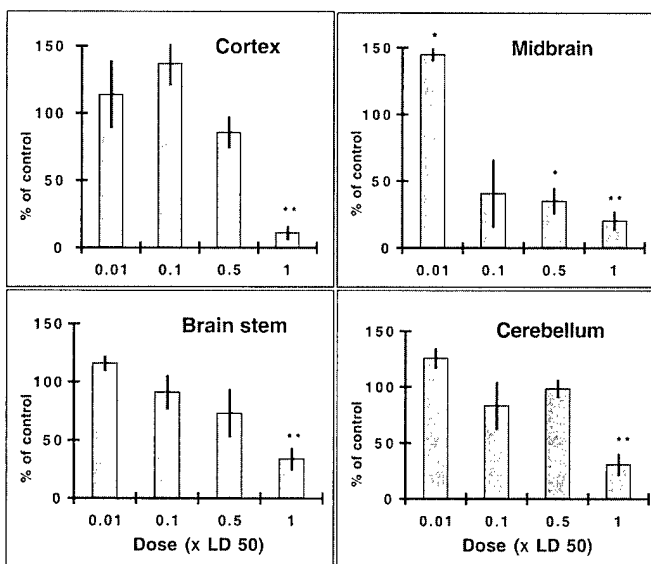


FIG. 4. Effect of sarin brain regional AChE activity, dose effect. Animals were treated with im injections of 0.01 , 0.1 , 0.5 , or $1 \times LD_{50}$ of sarin in saline and sacrificed after 15 h. Data are presented as mean \pm SE (% of control); * $p < 0.01$, ** $p < 0.001$.

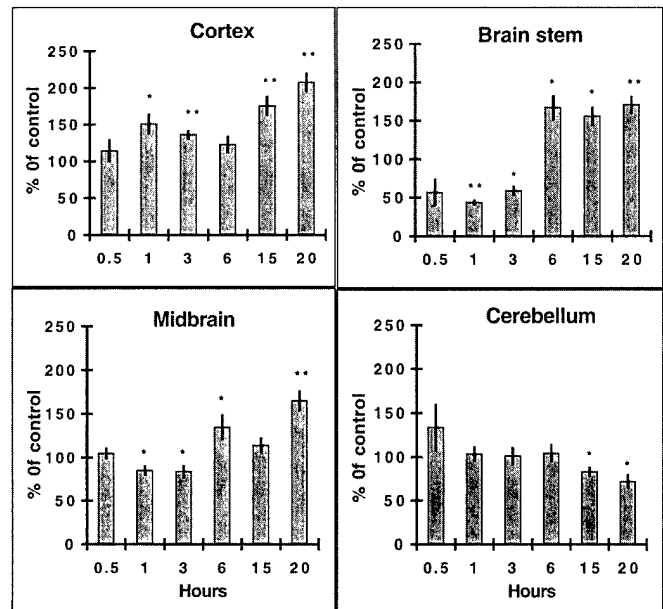


FIG. 5. Effect of sarin on brain regional choline acetyltransferase activity, time course. Animals were treated with single doses of sarin $1 \times LD_{50}$ ($100 \mu\text{g/kg}$, im) and brain regions were dissected at the indicated time period. The enzyme activity was determined as described in Materials and Methods. Data are presented as mean \pm SE (% of control); * $p < 0.01$; for cerebellum * $p < 0.02$; for midbrain * $p < 0.05$, ** $p < 0.001$.

brain showed a significant decrease in AChE activity at $0.1 \times LD_{50}$ ($\sim 60\%$ of control) and continued to decrease at 0.5 and $1 \times LD_{50}$. Cerebellar AChE activity was decreased (70%) significantly only at $1 \times LD_{50}$. At the $0.01 \times LD_{50}$ dose, midbrain, brainstem, and cerebellum showed consistent increases in AChE activity. It is apparent from these data that the dose response to sarin is variable in different regions of the rat brain.

Effect of Sarin on Brain Regional Choline Acetyltransferase (ChAT)

We carried out a time-course study on the effect of sarin on ChAT activity in different brain regions after a single LD_{50} exposure. Data presented in Figure 5 show that sarin treatment caused a significant increase in enzyme activity in the cortex at all the post-treatment time points. By 3 h after treatment, there was a significant increase ($\sim 138\%$ of control) in cortex activity, and it continued to rise for up to 20 h post-treatment. The brainstem activity showed a biphasic response: a decrease at early time points but a significant increase (~ 160 – 170% of control) by 6 to 20 h. These data suggest a differential regulation of ChAT activity in 2 brain regions that exhibit maximum cholinergic innervation. In midbrain, the activity followed a pattern similar to that in the brainstem. The cerebellar activity, even though not a main cholinergic response area, showed a declining trend.

Data from the dose study on ChAT activity are shown in

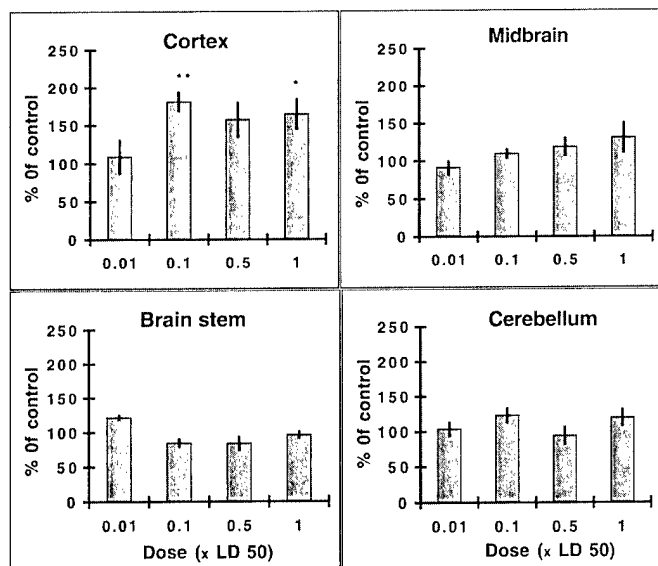


FIG. 6. Effect of sarin on brain regional choline-acetyltransferase activity, dose effect. Animals were treated with im injections of 0.01, 0.1, 0.5, or $1 \times LD_{50}$ of sarin in saline and sacrificed after 15 h. Data are presented as mean \pm SE (% of control); * $p < 0.04$, ** $p < 0.01$. Other values are not statistically significant.

Figure 6. As with the time course with a single LD_{50} dose study, the cortex area showed a significant increase (181, 157, and 164%) in enzyme activity after 0.1, 0.5, and $1 \times LD_{50}$, respectively. A similar pattern of activation was observed in mid brain.

Effect of Sarin on Nicotinic and m2-Muscarinic Acetylcholine Receptors in the Cortex

Sarin ($1 \times LD_{50}$) exposure resulted in a decrease ($\sim 25\%$) and an increase ($\sim 132\%$) in binding of [3H]cytisine 1 and 6 h after treatment, respectively. Increase in binding density persisted up to 20 h after treatment (Fig. 7). Furthermore, sarin caused an increase in the ligand-binding density at 0.01 of the LD_{50} dose. Because $\alpha 4\beta 2$ is the major receptor type present in the CNS of rats, our results suggest that sarin acutely regulates the nAChR ligand binding (Figs. 7 and 8).

The data presented in Figures 9 and 10 show that sarin treatment resulted in an initial decrease at 1 and 3 h post-treatment and subsequent increase in binding in the m2 m-AChR selective ligand 6 h after treatment of the LD_{50} sarin dose. Unlike nAChR, however, the increased binding in mAChR was only modest (10–15%) but persisted for up to 20 h after sarin treatment. Figure 10 shows the effect of various doses of sarin on m2-mAChR. The increase in binding density was observed at $0.01 \times LD_{50}$ dose. These data suggest that sarin exposure causes a persistent increase in receptor binding for nAChR, whereas m2-mAChR shows a different pattern of regulation.

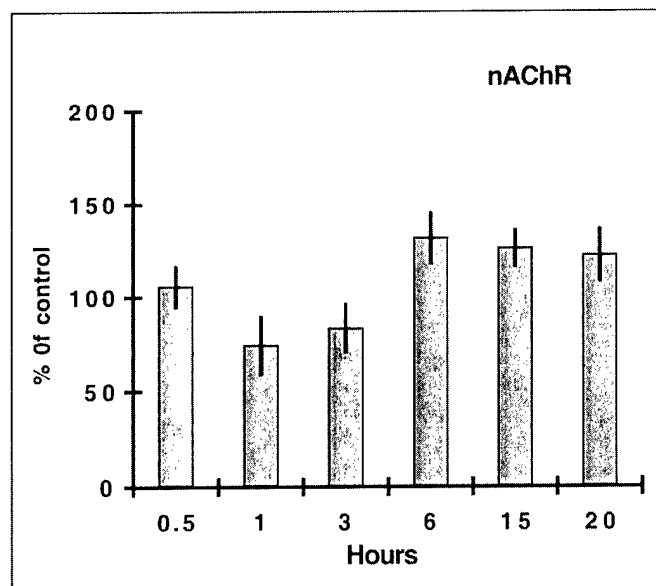


FIG. 7. Effect of sarin on cortex nAChR ligand binding, time course. Membranes were prepared and [3H]cytisine binding was carried out as described in Materials and Methods. Animals were treated with a single dose of sarin $1 \times LD_{50}$ ($100 \mu\text{g/kg}$, im) and brain regions were dissected at the indicated time periods. Data are presented as mean \pm SE (% of control).

DISCUSSION

The present study demonstrates that a single im dose of sarin, either at LD_{50} or less, induces changes in the CNS characteristic of organophosphate nerve agent toxicity. Besides the major clinical symptoms such as convulsions, fasciculations, tremors, and seizure, sarin treatment resulted in signifi-

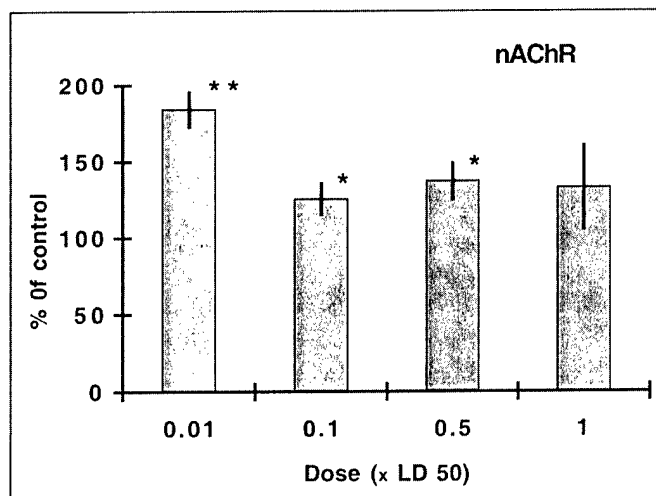


FIG. 8. Effect of sarin on cortex nAChR ligand binding, dose effect. Membranes were prepared and [3H]cytisine binding was carried out as described in Materials and Methods. Animals were treated with im injections of 0.01, 0.1, 0.5, or $1 \times LD_{50}$ of sarin in saline and sacrificed after 15 h. Data are presented as mean \pm SE (% of control); * $p < 0.05$, ** $p < 0.001$.

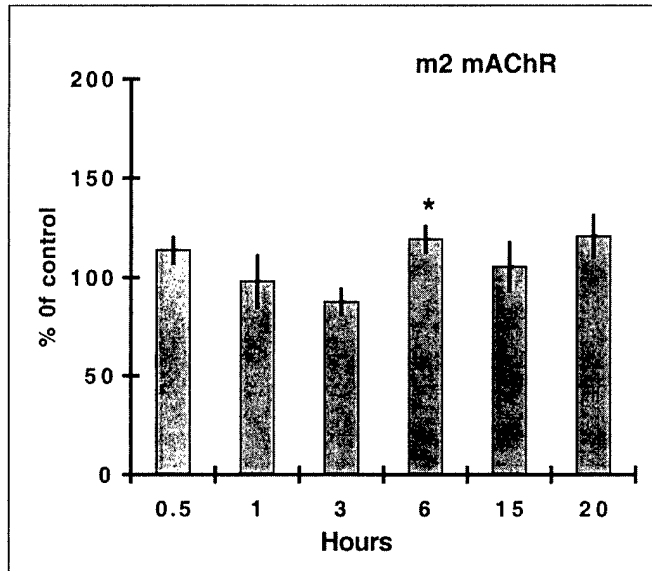


FIG. 9. Effect of sarin on cortex m2 mAChR ligand binding, time course. Membrane preparation and m2-mAChR-specific ligand binding with [3 H]AF-DX-384 was carried out as described in Materials and Methods. Animals were treated with a single dose of sarin $1 \times LD_{50}$ (100 μ g/kg, im) and brain regions were dissected at the indicated time periods. Data are presented as mean \pm SE (% of control); * $p < 0.02$.

cant inhibition in cholinesterase activity both in plasma and brain regions. The acute symptoms observed were a result of excessive cholinergic activity resulting from the accumulation of ACh at the synapse. Our data also provide evidence that activation of ChAT in cortex, brainstem, and midbrain may enhance the availability of acetylcholine above and beyond that afforded by inhibition of AChE by sarin. This could lead to overstimulation of AChR in the CNS.

Several studies have reported inhibition of AChE by sarin (Lim *et al.*, 1983 and references therein). The regulatory role of sarin on biosynthesis and degradation of acetylcholine and its role on the CNS nAChR and mAChR, however, has not been reported in a single study. It is important to evaluate these aspects of the cholinergic pathway, because the interplay between each component of the cholinergic pathway would ultimately affect the neurotoxicity of sarin. The present study provides data on all of these aspects of sarin-induced toxicity.

Inhibition of plasma BChE is the hallmark of the neurotoxicity induced by a large number of organophosphates, including sarin. Our time-course study with $1 \times LD_{50}$ sarin dosage shows that sarin treatment resulted in a significant decrease in BChE activity in plasma and AChE inhibition in cortex, brainstem, midbrain, and cerebellum. The dose-response study showed that significant inhibition in BChE activity is observed only at the higher dose of sarin at 15 h of treatment. However, it is likely that lower doses such as 0.01 and $0.1 \times LD_{50}$ may also have an inhibitory effect at earlier time periods, which is subsequently recovered. It is known that sarin-inhibited AChE ages slowly (Clement, 1982), and therefore, the inhibition

might have subsided by 15 h, the time of our study. Increase in AChE activity at $0.01 \times LD_{50}$ sarin may be mediated by an indirect mechanism as opposed to the inhibition of AChE by sarin, which is by direct interaction with the enzyme.

Our results also suggest that there are regional differences in the brain severity to inhibition of AChE by lower doses of sarin, with midbrain showing inhibition of AChE activity at $0.1 \times LD_{50}$, whereas in the cortex and brainstem, the inhibition was observed at $0.5 \times LD_{50}$. This differential response could reflect the total enzymatic activity present in each region. Thus, the threshold level of cortex and other brain regions such as striatum AChE may be higher than in the rest of the regions.

ChAT is a specific marker of cholinergic innervation in the CNS, which catalyzes the final step in the biosynthesis of acetylcholine (Wu and Hersh, 1994). Although it is believed that ChAT is not the rate-limiting enzyme in the availability of acetylcholine in the CNS, it can have a modulatory role in the cholinergic system. Indeed, in the past attempts have been made to use selective inhibitors of choline acetyltransferase as possible *in vivo* protection mechanisms against soman-induced neurotoxicity (Harris *et al.*, 1982; Schoene *et al.*, 1977). Sterling *et al.* (1988) reported that a quaternary salt of hydroxyethyl naphthylvinyl pyridine provided protection against soman-induced mortality when given 2–3 min prior to soman treatment. Our data on ChAT activation by sarin in cortex and brainstem prove that enhanced enzyme activity may have some consequence, at least in the early period of exposure. Similarly, Brookes and Goldberg (1979), using mouse spinal cord cell culture, found that diisopropylphosphorofluoridate (DFP) exposure caused activation of ChAT. Others found no effect on

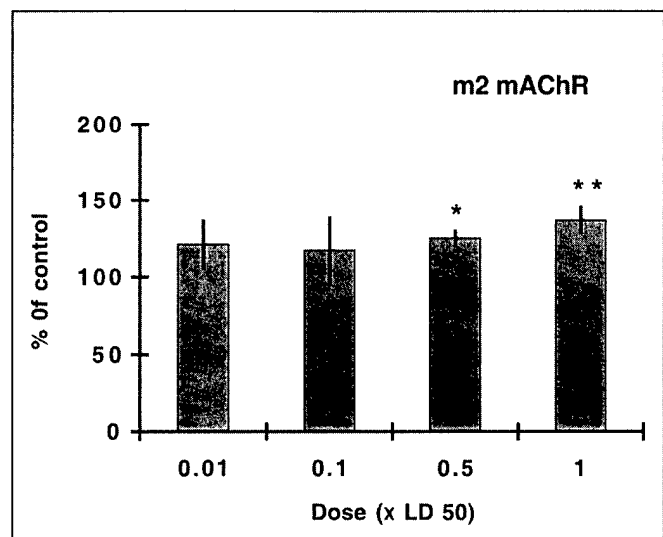


FIG. 10. Effect of sarin on cortex m2-mAChR ligand binding, dose effect. Membrane preparation and m2-mAChR-specific ligand binding with [3 H]AF-DX-384 were carried out as described in Materials and Methods. Animals were treated with im injections of 0.01, 0.1, 0.5, or $1 \times LD_{50}$ of sarin in saline and sacrificed after 15 h. Data are presented as mean \pm SE (% of control); * $p < 0.02$, ** $p < 0.01$.

the enzyme activity in response to sarin or DFP exposure (Sivam *et al.*, 1984). The reasons for these differences are not known. Significant inhibition of ChAT activity in the brainstem at early time periods following LD₅₀ sarin administration may suggest a direct inhibitory effect.

ChAT activation by sarin *in vivo* is interesting, because it is known that sarin-inhibited AChE is reactivated faster than other nerve agent-inhibited AChE (Clement, 1991; Clement *et al.*, 1991; Schoene, 1978). Clement (1982) reported that soman-inhibited AChE ages faster than a sarin-inhibited enzyme. Therefore, in view of persistent activation of ChAT, as observed in our studies, it is reasonable to assume that even when AChE inhibition by sarin is not pronounced (because of faster reactivation), the still higher level of acetylcholine could be available at the presynaptic terminals. However, the role of vesicular acetylcholine transporter remains to be evaluated under these conditions.

ChAT activation has been observed under a variety of conditions; mostly related to trophic factors and survival (Cavicholi *et al.*, 1991; Fusco *et al.*, 1989; Li *et al.*, 1995; Mobley *et al.*, 1985; Wu and Hersh, 1994). However, other modifications such as phosphorylation and proteolysis have also been shown to regulate the enzyme activity (Bruce and Hersh, 1989; Wu *et al.*, 1995). Sarin-induced activation of ChAT activity may involve proteolytic cleavage of the enzyme. An increase in electrical activity has been shown to cause increased proteolytic activity by. Furthermore, it has been shown that cholinergic stimulation causes protease(s) activation that leads to synapse loss in activity-dependent manner (Liu *et al.*, 1994). Therefore, it is likely that increased ChAT activity following sarin exposure may be a consequence of protease-mediated activation of the enzyme. This view needs further studies.

Inhibition of AChE following OP poisoning causes excessive stimulation of CNS AChRs. Under acute exposure conditions, overstimulation leads to seizure and chronic activation may lead to impairment of memory function (Taylor, 1985). A critical role of nAChR and mAChR has been implicated in all these processes (McGehee, 1999; Wess, 1996). Our data suggest that acute sarin exposure significantly increases binding densities of respective ligands for nAChR and m2-mAChR. Earlier *in vitro* studies (Bakry *et al.*, 1988) reported inhibition in binding of high affinity m2 receptor ligand [³H]CD to mAChR by several organophosphates, including sarin. This effect was found to be selective for mAChR as the inhibition for nAChR was not pronounced. A recent study extended these observations by reporting that not only neuronal type nAChR but also electric ray nAChR, which is very similar to muscular AChR structurally and pharmacologically, binds organophosphates of diverse structures (Katz *et al.*, 1997). Binding of these organophosphate compounds to the nAChR is believed to be at a site distinct from the ligand binding site and this binding induces desensitization of the receptor (Albuquerque *et al.*, 1997; Bakry *et al.*, 1988). Increased binding densities of $\alpha 4\beta 2$ -specific nAChR ligand in the cortex in the present study at

0.001 LD₅₀ suggest that if the concentration of sarin reaches a high enough level in the cell, it may cause allosteric changes in the receptor conformation, exposing higher ligand binding sites. Whether or not a high ligand binding state of the receptor continues to be active long enough or becomes desensitized remains to be discovered. Alternatively, the number and or function of the receptor increased by sarin can be mediated by second messengers such as c-AMP-dependent or independent mechanisms (Gurant *et al.*, 1993; Margiotta, 1987) or by changes in intracellular Ca²⁺ concentration. An interesting possibility could also involve upregulation of the nAChR as a consequence of hypothermia induced by sarin (Clement, 1991) because low temperature causes upregulation in surface expression of nAChR (Cooper *et al.*, 1999).

Muscarinic acetylcholine receptors in the CNS are comprised of 5 distinct classes of receptors (m1-m5). These receptors have distinct structural and pharmacological features and show differential cellular localization (Levey *et al.*, 1991). These receptors are coupled to different G-proteins to transduce cellular signaling from the cell surface. m2-mAChR is coupled to Gi protein, leading to inhibition of adenylate cyclase (Hulme *et al.*, 1990). Our laboratory has previously shown that chlorpyrifos oxon binds to m2-mAChR *in vitro* and inhibits cAMP accumulation (Huff and Abou-Donia, 1995; Huff *et al.*, 1994). Studies by Ward *et al.* (1993) and Silveira *et al.* (1990) also have shown that organophosphate compounds selectively regulate m2-mAChR ligand binding. The data in the current study showing increased m2-mAChR-specific ligand binding in cortex following sarin treatment suggest *in vivo* regulation. Similarly, studies by Chaudhuri *et al.* (1993) and Liu and Pope (1996) reported an increased m2-mAChR ligand binding in response to chlorpyrifos treatment. It has been previously shown that presynaptic m2-mAChR could regulate acetylcholine release via a feedback inhibitory mechanism (Marchi *et al.*, 1990; Raiteri *et al.*, 1984) and in rat striatal cells, paraoxon inhibited forskolin induced cAMP synthesis, an effect which was blocked by atropine (Jett *et al.*, 1991). These results suggest that selective effects of sarin on m2-mAChR may have modulatory effects on other processes, such as acetylcholine release, second messenger system, etc. that could influence the toxicity of sarin.

In summary, our results suggest that BChE activity in plasma remains inhibited up to 15–20 h following a single LD₅₀ dose of sarin, whereas the brain regional AChE shows differential response to sarin treatment. ChAT activity was induced in the cortex followed by midbrain and brainstem. The increased ChAT activation may cause persistent long-term sarin toxicity even after AChE activity has recovered. Furthermore, our results indicate that sarin caused increased nAChR and m2-mAChR binding in the cortex after 6, 15, and 20 h of single-dose LD₅₀ treatment. Finally, our data clearly suggest that sarin-induced neurotoxicity has multiple mechanisms. The eventual manifestation of sarin toxicity is primarily a dysregulation of the cholinergic system.

ACKNOWLEDGMENTS

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SUBCHRONIC EFFECTS FOLLOWING A SINGLE SARIN EXPOSURE ON BLOOD-BRAIN AND BLOOD-TESTES BARRIER PERMEABILITY, ACETYLCHOLINESTERASE, AND ACETYLCHOLINE RECEPTORS IN THE CENTRAL NERVOUS SYSTEM OF RAT: A DOSE-RESPONSE STUDY

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Subchronic neurotoxic effects of sarin (O-isopropyl methylphosphonofluoridate) treatment at various doses in male Sprague Dawley rats were studied. The animals were treated with a single intramuscular (im) injection of 0.01, 0.1, 0.5, or 1 × LD50 (100 µg/kg). The animals were maintained for 90 d thereafter. [³H]Hexamethonium iodide was used to monitor the changes in blood-brain barrier (BBB) permeability in cortex, brainstem, midbrain, and cerebellum. Brainstem exhibited a significant decrease (~58% of control) in uptake of [³H]hexamethonium iodide at 1 × LD50 dose. No significant changes were observed in BBB permeability in cortex, midbrain, and cerebellum at any dose. Plasma butyrylcholinesterase (BChE) activity remained unchanged, reflecting recovery of the enzyme activity from the initial inhibition following single exposure of 1 × LD50 sarin. Acetylcholinesterase (AChE) activity in the cortex remained inhibited (~29%), whereas in the brainstem there was an increase (~20%) at 1 × LD50 dose of sarin. The m2-selective muscarinic acetylcholine receptor (m2-mAChR) ligand binding was inhibited significantly at 1 × LD50 in the cortex, whereas brainstem showed significantly increased (~45%) ligand binding at 1 × LD50 dose. Nicotinic acetylcholine receptor (nAChR), on the other hand, showed a biphasic response in ligand binding in the cortex with a decrease (~30%) at 0.01 × LD50 but an increase (~40%) at 1 × LD50. Brainstem did not show any significant change in nAChR ligand binding. These results suggest that single exposure of sarin could lead to changes that may play an important role in neuropathological abnormalities in the central nervous system.

During the Gulf War several hundred thousand American Army personnel as well as army personnel from other countries were stationed in Persian Gulf region. Since their return, several thousand veterans and active duty personnel have complained of unexplained illnesses, including chronic fatigue, muscle and joint pain, ataxia, headache, difficulty concentrating, forgetfulness, and irritability (Persian Gulf Veterans Coordi-

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nation Board, 1995). Because these Persian Gulf War veterans were exposed to a unique combination of biological, chemical, and environmental conditions, it is likely that some of the complaints reported may have been caused by the constituents of biological and chemical warfare either alone or in combination with other factors. In light of this, recent reports have indicated that thousands of army personnel might have been exposed to the nerve agent sarin during the Gulf War (Institute of Medicine, 1995). Some of these veterans complained of symptoms that involve defects in, or abnormal regulation of, the central and peripheral cholinergic nervous systems (Persian Gulf Veterans Coordination Board, 1995).

Sarin, *O*-isopropyl methylphosphonofluoridate, was developed as a warfare nerve agent (Wood, 1951). The neurotoxicity of sarin has been evaluated in different rodent and mammalian species, and it is well accepted that the acute toxicity is mediated by inhibition of acetylcholinesterase (AChE). The main clinical features associated with acute sarin intoxication are seizures, fasciculations, tremors, and hypothermia (Taylor, 1985). The appearance of these symptoms correlates with the inhibition of AChE, both in the central (CNS) and peripheral (PNS) nervous systems (Gupta et al., 1991). This is followed by increased accumulation of acetylcholine, which leads to hyperactivation of nicotinic and muscarinic acetylcholine receptors.

Excessive accumulation of acetylcholine leads to activation of the ligand-gated ion channels, nicotinic acetylcholine receptors (nAChR), and muscarinic acetylcholine receptor (mAChR). These receptors activate diverse kinds of cellular responses by distinct signaling mechanisms (Wess, 1996). *In vitro* studies by Bakry et al. (1988) suggested that sarin binds to nAChR and modulates ligand binding characteristics. In addition to the acute toxic effects of sarin, long-term neurological dysfunctions have been observed. Signs of neurological dysfunctions have been observed in humans 10 yr after low-dose sarin exposure (Sidell, 1974; Duffy & Burchfiel, 1980). A single large-dose sarin exposure has been found to cause neuropathy in rats (Kadar et al., 1995; Lemerrier et al., 1983). The neuropathological changes induced by sarin were characterized by persistent brain lesions. These changes occurred mainly in the hippocampus, cortex, and thalamic nuclei (Kadar et al., 1995). The pathological changes were observed as early as 24 h and 7 d and persisted up to 90 d following sarin treatment. Abnormal CNS functions in rhesus monkeys (Burchfiel et al., 1976; Burchfiel & Duffy, 1982) have also been reported following repeated subclinical doses of sarin. In the present study, therefore, it was decided to carry out a dose-response study of a single exposure sarin at 0.01, 0.1, 0.5, and 1 \times LD50, to evaluate the subchronic effects after 90 d of treatment. These results suggest that blood-blood barrier (BBB) changes, inhibition of AChE in the cortex, and inhibition in m2-mAChR and nAChR in the cortex are persistent following 90 d of 1 \times LD50 sarin exposure.

MATERIALS AND METHODS

Materials

Butyrylcholine iodide, acetylcholine iodide, acetyl coenzyme A (CoA), and choline chloride were obtained from Sigma Chemical Co. (St. Louis, MO). [^3H]Cytisine (specific activity 15 Ci/mmol) and [^3H]AF-DX384[2,3 dipropylamino] (specific activity, 100 Ci/mmol) was obtained from NEN (Boston). [^3H]Hexamethonium iodide, specific activity 18 Ci/mmol was obtained from USAMRID, Aberdeen Proving Ground, MD. All other reagents were of highest purity available commercially. Sarin (1.9 mg/ml in saline) was obtained from Fort Detrick Army Headquarters, MD.

Male Sprague-Dawley rats (200–250 g) were obtained from Zivic-Miller Laboratories (Allison Park, PA) and housed in the Duke University Medical Center vivarium on a 12-h dark–light cycle. The animals were allowed food and water ad libitum. The animals were randomly assigned to control and treated groups. Each group consisted of a minimum of five surviving animals at the termination of the experiment. All the treatments and procedures of the animals were carried out strictly according to the recommended guidelines by the Army and the Duke University institutional animal care and use committee.

Treatment of Animals and Tissue Retrieval

Animals were treated with single intramuscular injections of sarin in the thigh muscle with 0.01, 0.1, 0.5, or $1 \times \text{LD}_{50}$ (100 $\mu\text{g/kg}$) in normal saline. Control animals received an equal volume of vehicle. The animals were maintained for 90 d thereafter. At the termination of the experiment, the animals were anesthetized with 0.2 ml ketamine/xylazine, and blood was drawn into a heparinized syringe. Animals were dissected; the brain was removed and washed thoroughly with ice-cold normal saline to remove traces of blood. Brain regions, cortex, midbrain, cerebellum, and brainstem were dissected on ice and snap frozen in liquid nitrogen.

For blood–brain barrier (BBB) studies, after 90 d of sarin treatment the animals were anesthetized and then injected with [^3H]hexamethonium iodide (10 μCi , mixed with cold hexamethonium iodide) to give a final dose of 0.7 mg/kg (1 $\mu\text{Ci/kg}$) in the tail vein. After 10 min the blood was collected from the heart in heparinized syringes and animals were sacrificed by decapitation. Brains and testes were removed and washed with ice-cold saline. Brain regions were dissected on ice into cortex, brainstem, midbrain, and cerebellum and frozen in liquid nitrogen and stored at -70°C until use. Plasma was separated from whole blood by centrifugation.

Total radioactivity in tissues and plasma was analyzed in triplicate in a Beckman LS-6500 multipurpose scintillation spectrometer (Beckman Instruments Corp., Palo Alto, CA) following combustion in a Packard 306B tissue oxidizer (Packard Instrument Co., Downers Grove, IL).

Enzyme and Receptor Assay

Cholinesterase Determination Activities of AChE, in brain regions, and BChE, in plasma, were determined according to the method of Ellman et al. (1961) modified for assay in a Molecular Devices UV Max kinetic microplate reader as previously described (Abou-Donia et al., 1996). In brief, brain regions were weighed and 10% homogenate was prepared in 0.1 M phosphate buffer, pH 8, containing 0.5% Triton X-100. The homogenate was centrifuged at $5000 \times g$ for 10 min at 4°C. The supernatant was used as the source of the enzyme. Blood samples were centrifuged at $5000 \times g$ for 10 min to separate plasma. All samples were stored at -70°C until use. All tissue supernatants were diluted 1:10 with phosphate-buffered saline (PBS) containing 10 mM MgCl₂, pH 7. Twenty microliters of diluted supernatant was used for each assay in a total volume of 200 µl of buffer or 0.2 mM acetylthiocholine iodide. For plasma BChE determination, the plasma was diluted 1:10 in PBS containing 10 mM MgCl₂ and assayed as described for brain regions except that 0.2 mM butyrylthiocholine (BSCh) was used as substrate in the presence of 5×10^{-7} M AChE inhibitor, 1,5-bis-(*N*-allyl-*N*-dimethyl-4-ammoniumphenyl) pentane-3-one dibromide. The reaction was started by the addition of 0.1 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in PBS. Blank contained buffer in the place of substrate. The enzyme activity was monitored by recording the absorbance at 412 nm. Protein concentration was determined by bicinchoninic acid (BCA) method according to Smith et al. (1985). The enzyme activities are expressed as micromoles substrate hydrolyzed per minute per milligram protein for brain regions and nanomoles substrate hydrolyzed per minute per milligram protein for plasma.

Nicotinic Acetylcholine Receptor (nAChR) Binding Assay [³H]-Cytisine was used as specific ligand for binding studies with nAChR according to the method described by Slotkin et al. (1999). The tissue was homogenized in 50 mM Tris-HCl, pH 7.4, containing 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂ and 2.5 mM MgCl₂. The membranes were sedimented by centrifuging at $40,000 \times g$ for 10 min. The resulting membrane pellet was resuspended in the same buffer using a Teflon pestle glass homogenizer in a volume to give 1.5 to 2 mg/ml protein. An aliquot of membrane preparation containing ~200 µg protein was used to carry out the incubation with 1 nM [³H]cytosine at 4°C for 75 min. Nonspecific binding was carried out in presence of 1 µM nicotine ditartrate. The labeled membranes were trapped on membrane filters using rapid vacuum filtration system and the results are expressed as specific binding per milligram protein.

Muscarinic Acetylcholine Receptor (mAChR) Binding Assay For the assay of mAChR, the tissue was homogenized in 10 mM phosphate buffer, pH 7.4, centrifuged at $40,000 \times g$ for 10 min, and the membranes were suspended in the same buffer at the protein concentration of 1.5–2.5 mg/ml as described by Huff et al. (1994). The m2-mAChR binding

was carried out according to the method of Slotkin et al. (1999) by using the m2-selective ligand [^3H]AFDX 384 at room temperature for 60 min. Nonspecific binding was carried out in the presence of 2.22 μM atropine. Ligand-bound membranes were trapped on glass filters presoaked with 0.1% polyethyleneimine using rapid vacuum filtration as described for the nAChR assay. The results are expressed as specific binding per milligram protein.

Statistical Analysis

The data were analyzed by Student's *t*-test for the level of significance. The graphs were generated on Excel graphics for Macintosh and presented as mean \pm SE (% of control).

RESULTS

Clinical Signs

All animals were observed for the development of clinical signs of toxicity. Animals treated with sarin at $1 \times \text{LD}_{50}$ showed severe tremors, seizures, and salivation within 5–15 min of treatment and continued with increasing severity up to 30 min. Acute death occurred in some animals that exhibited severe convulsions possibly because of respiratory paralysis. All the animals treated with sarin who survived the first hour after the treatment remained alive till the termination of the experiment.

Effect of Single Exposure of Sarin on Blood–Brain Barrier Permeability in Brain Regions and Testes After 90 d of the Treatment

Effects of single im injections of 0.01, 0.1, 0.5, and $1 \times \text{LD}_{50}$ (100 $\mu\text{g}/\text{kg}$) on blood–brain barrier (BBB) permeability are shown in Figure 1. [^3H]Hexamethonium iodide was used to monitor the BBB changes in cortex, brainstem, midbrain, and cerebellum, and permeability changes in testes. Brainstem exhibited significant decrease in permeability ($\sim 58\%$ of control) at $1 \times \text{LD}_{50}$ ($p < .03$). No significant changes were observed in cortex, midbrain, and cerebellum. An increase ($\sim 30\%$) in barrier permeability was observed in testes at 0.1 and $1 \times \text{LD}_{50}$ doses, but these values were not significant because of variation in response within the group treated with sarin. However, no changes were observed at $0.5 \times \text{LD}_{50}$ dose.

Effect of Single Exposure of Sarin on Cholinesterase Activities in Brain Regions and Plasma After 90 d of the Treatment

The data presented in Figure 2 show the effect of single im injections of 0.01, 0.1, 0.5, and $1 \times \text{LD}_{50}$ (100 $\mu\text{g}/\text{kg}$) on brain regional acetylcholinesterase (AChE) and plasma butyrylcholinesterase (BChE) activities. No significant changes were observed in cortex and brainstem AChE activity at 0.01, 0.1, or $0.5 \times \text{LD}_{50}$. Cortex AChE was inhibited ($\sim 30\%$)

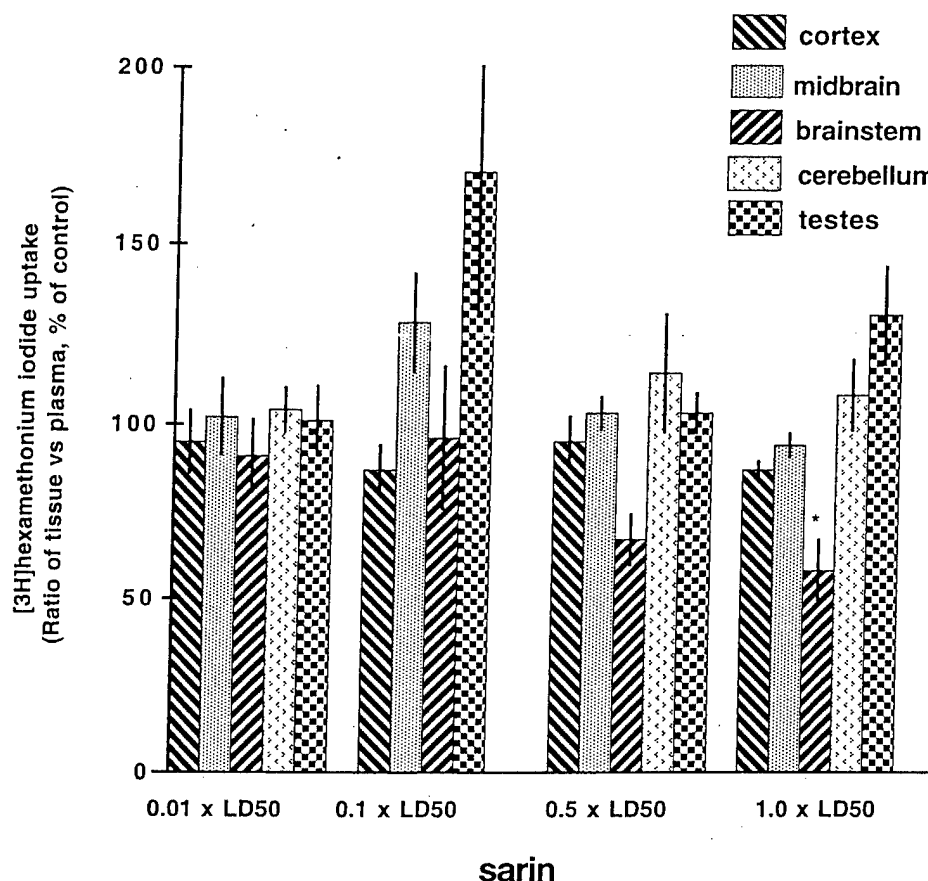


FIGURE 1. Effect of sarin on [^3H]hexamethonium iodide uptake in brain regions and testes following single exposure of sarin. Animals were treated with single im injections of 0.01, 0.1, 0.5, and 1 \times LD50 (100 $\mu\text{g/kg}$) of sarin in saline and maintained for 90 d thereafter. The animals were given [^3H]hexamethonium iodide iv under anesthesia. Ten minutes after the [^3H]hexamethonium iodide injection, the blood was collected, brain regions and testes were dissected, and radioactivity was measured as described in Materials and Methods. Data are presented as mean \pm SE of percent control of the ratio between tissue uptake versus that of plasma. Asterisk indicates statistically significant at $p < .03$.

but was not significant because of high variability in the activity in sarin treated animals ($p < .2$). The enzyme activity in brainstem was increased ($\sim 20\%$, $p < .02$) after 1 \times LD50 dose of sarin treatment. Plasma BChE activity did not show any changes.

Effect of Single Exposure of Sarin on Brain Regional m2-Muscarinic Acetylcholine Receptors After 90 d of the Treatment

The data presented in Figure 3 show the effect of a single im injection of sarin on cortex and brainstem m2-mAChR-selective ligand binding. Cortex ligand binding was significantly reduced at 0.5 and 1 \times LD50 in a

dose-dependent manner (~33% and 61%, $p < .08$ and $p < .009$, respectively). Receptor binding in the brainstem, however, was significantly increased at $1 \times \text{LD}_{50}$ (~45%, $p < .01$).

Effect of Single Exposure of Sarin on Brain Regional Nicotinic Acetylcholine Receptors After 90 d of the Treatment

Effects of single im injections of sarin at 0.01, 0.1, 0.5, and $1 \times \text{LD}_{50}$ on cortex and brainstem nicotinic acetylcholine receptor (nAChR) are presented in Figure 4. nAChR ligand binding in the cortex showed a biphasic response. The receptor binding was increased ~30%, ~20%, and ~45% at 0.01, 0.1, and $0.5 \times \text{LD}_{50}$, respectively, whereas there was a significant decrease (~40%) at $1 \times \text{LD}_{50}$ dose. Brainstem, on the other hand, did not show any significant change.

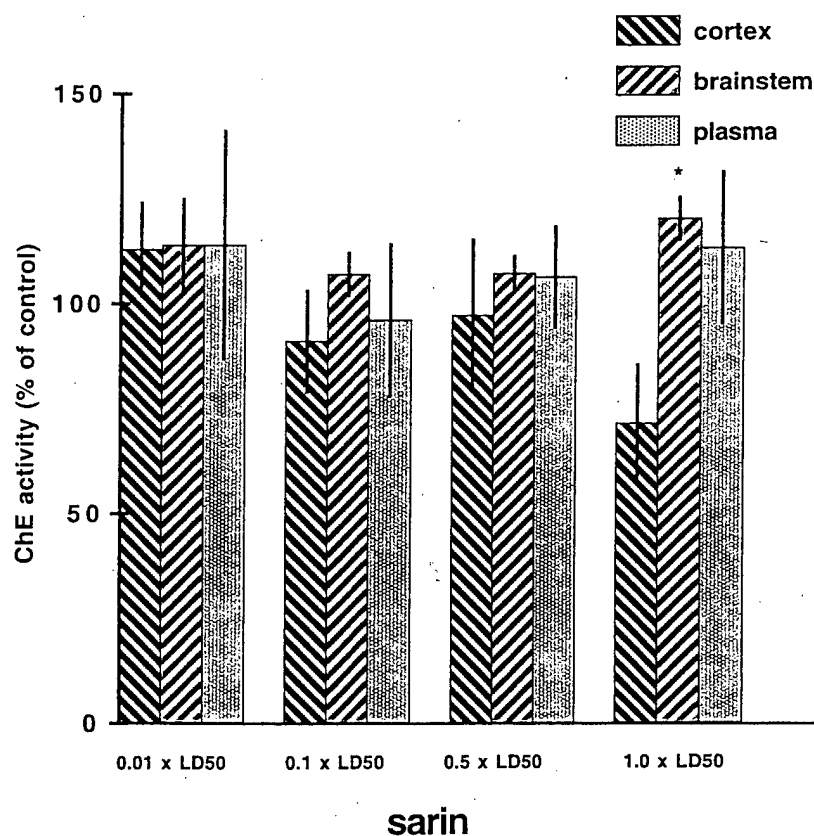


FIGURE 2. Effect of sarin on plasma BChE brain regional AChE activity. Animals were treated with single im injections of 0.01, 0.1, 0.5, and $1 \times \text{LD}_{50}$ (100 $\mu\text{g}/\text{kg}$) of sarin in saline and maintained for 90 d thereafter. Animals were sacrificed after 90 d, and plasma and brain region enzyme activities were determined as described in Materials and Methods. Data are presented as mean \pm SE (percent of control). Asterisk indicates significant at $p < .02$.

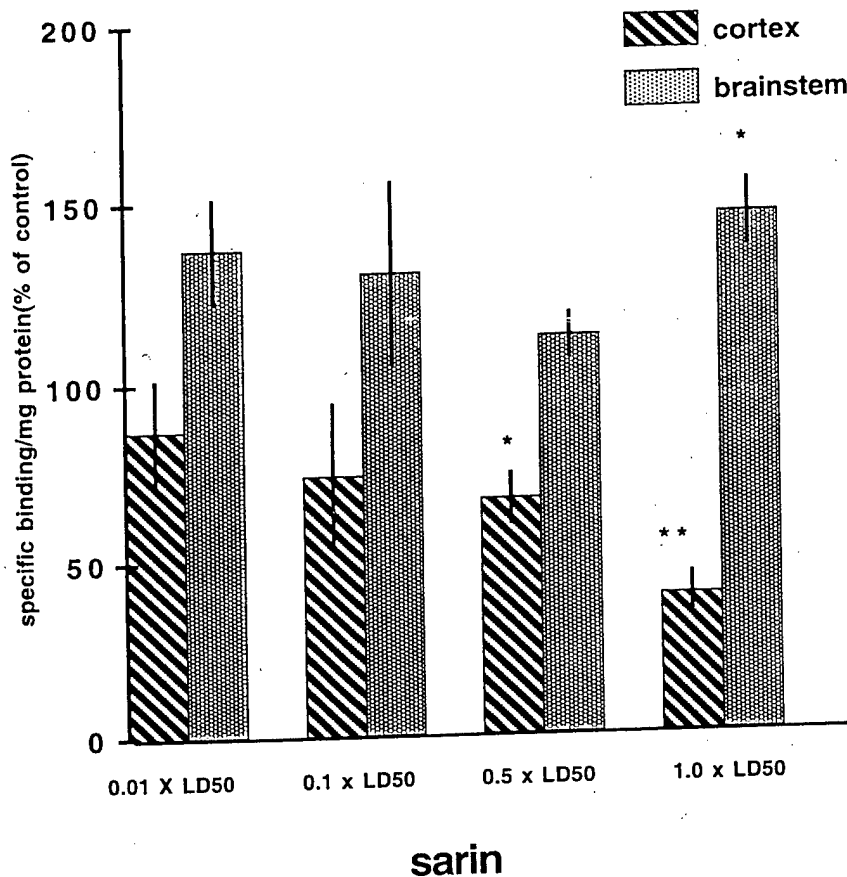


FIGURE 3. Effect of sarin on m2-mAChR ligand binding. Animals were treated with single im injections of 0.01, 0.1, 0.5, and 1 \times LD50 (100 μ g/kg) of sarin in saline and maintained for 90 d thereafter. Animals were sacrificed after 90 d and brain regions were dissected. Membrane preparation and m2-mAChR-specific ligand binding with [3 H]AF-DX-384 was carried out as described in Materials and Methods. Data are presented as mean \pm SE (percent of control). Asterisk indicates significant at p for cortex $< .08$, double asterisk, $p < .009$; brainstem, asterisk indicates significant at $p < .01$.

DISCUSSION

The present study was designed to evaluate the long-term toxic effects of a single exposure of sarin at various doses ranging from 1/100 to 1 \times LD50. The results presented in these studies suggest that even though rats recovered from all of the acute clinical effects of sarin at 1 \times LD50, there are changes in the central nervous system (CNS) that may play an important role in the neuropathological conditions long after the sarin exposure has ceased. Indeed, it has been reported that healthy individuals exposed to low levels of sarin continued to exhibit signs and symptoms of neurological dysfunctions up to 10 yr after the initial exposure (Sidell, 1974; Duffy & Burchfiel, 1980). Furthermore, abnormal electrophysiological

recordings following a single large dose or repeated subclinical dose of sarin in rhesus monkeys (Burchfiel et al., 1976; Burchfiel & Duffy, 1982) and neuropathy in rats (Lemercier et al., 1983; Kadar et al., 1995) have been observed.

The BBB is the interface between blood and the CNS that regulates the entry of molecules to the CNS based on the size, charge, hydrophobicity, and/or affinity to carriers. Because of the selective nature of the BBB, it helps to maintain the homeostasis of the CNS environment to ensure proper function (Joo, 1996). The BBB consists of the cerebral capillary endothelium, which contains tight junctions that form rows of extensive overlapping occlusions that block the intercellular route of solute entry that might alter the neuronal functions. Any changes in the basal permeability of the cerebral capillary endothelium can exacerbate a variety of patho-

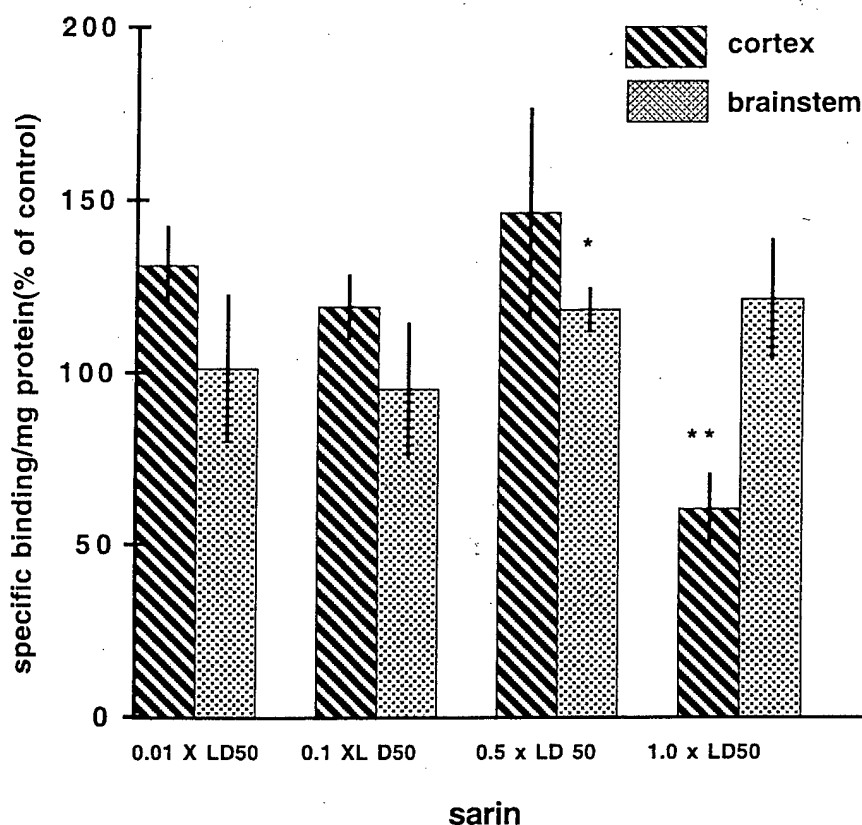


FIGURE 4. Effect of sarin on nAChR ligand binding. Animals were treated with single im injections of 0.01, 0.1, 0.5, and 1 \times LD50 (100 μ g/kg) of sarin in saline and maintained for 90 d thereafter. Animals were sacrificed after 90 d and brain regions were dissected. Membranes were prepared and [3 H]cytisine binding was carried out as described in Materials and Methods. Data presented as mean \pm SE (percent of control). Asterisk indicates significant at $p < .05$; double asterisk, $p < .03$.

logical processes. Our finding that 90 d after treatment, sarin exposure at $0.5 \times \text{LD}_{50}$ or $1 \times \text{LD}_{50}$ caused a decrease in the BBB permeability in brainstem and at $1 \times \text{LD}_{50}$ in the cortex indicates that this may restrict passage of important molecules that are required for normal homeostasis in the CNS. The decrease in BBB permeability could be mediated by one of the several mechanisms; for example, it is known that membrane fluidity changes induced by ethanol cause a decrease in the passage of [^3H]-vincristine to the CNS (Domer & Smith, 1988). Increased cAMP levels have been shown to reduce the BBB permeability induced by cerebral ischemia (Belayev et al., 1998). It is possible that sarin could modulate the levels of cAMP after prolonged exposure. Acute sarin exposure induces hypothermia, which is known to reduce BBB permeability. Additionally, sarin could modulate the surface expression of tight gap junction proteins in such a way that it causes a decrease in blood flow. Decrease in BBB permeability in brainstem and cortex following treatment with sarin could result by any, all, or a combination of the mechanisms just discussed. In addition, our results also suggest that sarin exposure could lead to an increase in blood-testes barrier permeability, possibly causing persistent changes in testicular functions. Therefore, it is possible that long-term effects of sarin may include abnormal reproductive functions.

Inhibition of plasma BChE and CNS AChE is the hallmark of the neurotoxicity induced by a large number of organophosphate agents, including sarin. In our previous studies, it was reported that acute sarin exposure at $1 \times \text{LD}_{50}$ resulted in a significant decrease in BChE activity in plasma and AChE inhibition in cortex, brainstem, midbrain, and cerebellum (Khan et al., 2000). The data in the present studies suggest that even though sarin-inhibited AChE activity recovers rapidly, the cortex activity remains slightly inhibited after 90 d following single sarin treatment. The level of acetylcholine in the CNS is controlled by AChE in a fine-tuned manner because of its extremely potent activity (Koelle, 1994). Long-term changes (inhibition or increased activity) in brain regions may be detrimental to the functions associated with the affected region. Furthermore, it is known that sarin-inhibited AChE ages slowly (Clement, 1982; Lim et al., 1983; Schoene, 1978), and therefore the inhibition might have subsided, and instead the enzyme activity may have recovered in brainstem to such an extent that there is an increase after 90 d of a single treatment. This increase in AChE activity may be mediated at the transcriptional level, as has been reported recently (Friedman et al., 1996), in response to stress and pyridostigmine treatment.

Our results also suggest that there are regional differences in the brain recovery of AChE following inhibition by sarin such that brainstem shows an increase at all doses, whereas in the cortex a moderate inhibitory response persists after long-term single exposure. Inhibition of AChE following organophosphate (OP) poisoning causes excessive stimulation of CNS AChRs. Under acute exposure conditions, overstimulation leads to sei-

zures, and chronic activation may lead to impairment of memory functions (Taylor, 1985). A critical role of nAChR and mAChR has been implicated in all of these processes (Wess, 1996; McGehee, 1999). Our data suggest that following a single exposure to sarin there is significant increase in binding densities of m2-mAChR and a moderate increase in nAChR in the brainstem. In contrast, m2-mAChR binding was significantly decreased in the cortex and the response to nAChR was inconsistent; there was an increase at lower doses but a significant decrease at $1 \times \text{LD}_{50}$. Earlier in vitro studies (Bakry et al., 1988) reported inhibition in binding of the high-affinity m2 receptor ligand *cis*-methyldioxolane ($[^3\text{H}]$ -CD) to mAChR by several organophosphates, including sarin. This effect was found to be selective for mAChR, as the inhibition for nAChR was not pronounced. A recent study extended these observations by reporting that not only neuronal type nAChR but also electric ray nAChR, which is very similar to muscle AChR structurally and pharmacologically, binds organophosphates of diverse structures (Katz et al., 1997; Ward et al., 1993). Binding of these organophosphate compounds to the nAChR is believed to be at a site distinct from the ligand binding site inducing desensitization of the receptor (Bakry et al., 1988; Albuquerque et al., 1997). A decrease in the ligand binding at $1 \times \text{LD}_{50}$ may reflect desensitization of the receptor, so that the ligand binding sites are no longer exposed, or a high turnover rate of the receptor.

Muscarinic acetylcholine receptors in the CNS comprise five distinct classes of receptors (M1–M5). The receptors have distinct structural and pharmacological features and show differential cellular localization (Levey et al., 1991). Some of these receptors (m2 and m4) are coupled to different G proteins to transduce cellular signaling from the cell surface. m2-mAChR is coupled to inhibitory G protein, G_i , leading to inhibition of adenylate cyclase (Hulme et al., 1990). Our laboratory has previously shown that chlorpyrifos oxon binds to m2-mAChR in vitro and inhibits cAMP accumulation (Huff et al., 1994; Huff & Abou-Donia, 1995). Studies by Ward et al. (1993) and Silviera et al. (1990) also have shown that m2-mAChR is selectively regulated by organophosphate compounds. Data in the current study showing increased m2-mAChR-specific ligand binding in the brainstem at $1 \times \text{LD}_{50}$ and a dose-dependent decrease in cortex following sarin treatment suggest differential in vivo regulation. These changes may play an important role in long-term CNS toxicity following sarin exposure. It has been previously shown that presynaptic m2-mAChR could regulate acetylcholine release via a feedback-inhibitory mechanism (Marchi et al., 1990; Raiteri et al., 1984). In rat striatal cells, paraoxon also inhibited forskolin-induced cAMP synthesis, an effect that was blocked by atropine (Jett et al., 1991).

In summary, our results suggest that BChE activity in plasma rapidly recovers and therefore can not be a reliable biomarker for long-term toxicity of sarin exposure, whereas brain regional AChE shows differential

chronic responses to a single sarin treatment. Furthermore, our results indicate that 90 d after a single exposure to sarin, there is an increase in ligand binding densities of m2-mAChR in the brainstem. There was a decrease in m2-mAChR in the cortex and a biphasic response in nAChR in the cortex. The long-term pathological changes following a single sarin exposure may be related to brain-region-specific alterations in these molecular targets.

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Combined exposure to sarin and pyridostigmine bromide increased levels of rat urinary 3-nitrotyrosine and 8-hydroxy-2'-deoxyguanosine, biomarkers of oxidative stress

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Abstract

In this study concentrations of markers of oxidative stress 3-nitrotyrosine and 8-hydroxy-2'-deoxyguanosine (8-OHdG) were determined in rat urine following a single oral dose of pyridostigmine bromide (PB) 13 mg/kg and a single intramuscular dose of sarin 80 µg/kg alone or in combination. Urine samples were collected 16, 24, 48, 72, and 96 h following dosing. Control urine samples of five rats treated with normal saline were also collected at the same time intervals. A combined dose of PB and sarin significantly increased levels of 3-nitrotyrosine and (8-OHdG) starting 48 h after dosing. An increase in the concentration of these markers was not detected following a single dose of PB or sarin alone. Maximal increase in 3-nitrotyrosine and 8-OHdG was detected 48 h after administration of a combination PB and sarin. The results indicate that concurrent exposure to PB and sarin could generate free radical species that may cause oxidative stress in rats. The results may have significant impact if veterans were exposed to sarin following an oral dose of PB. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Pyridostigmine bromide; Sarin; Oxidative stress; Nerve agents

1. Introduction

Sarin is a highly toxic nerve agent produced for chemical warfare (Somani, 1992; Bakshi et al., 2000). People were exposed to sarin inadvertently during the Gulf War (Institute of Medicine, 2000), and in Japan as a result of deliberate attacks (Nakajima et al., 1998). Neurotoxicity from sarin

exposure is mediated by inhibition of acetylcholinesterase (AChE) (Khan et al., 2000). The main clinical symptoms of acute toxicity of sarin are seizure, weight loss, tremors, and hypothermia (Bakshi et al., 2000; Young et al., 2001). These symptoms were correlated with inhibition of (AChE), both in the central and peripheral nervous system (Jones et al., 2000; Khan et al., 2000). Pyridostigmine bromide (PB) has been used in the treatment of myasthenia gravis patients, and may be used following surgery in the reversal of neuromuscular blockade (Aquilonius and Hartvig, 1986), and it is applied as a prophylactic agent

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against nerve agents such as sarin (Abou-Donia et al., 1996; Institute of Medicine, 2000). PB inhibits (AChE) and butyrylcholinesterase (BuChE) enzymes in the peripheral nervous system (Abou-Donia et al., 1996; Somani et al., 2000).

Oxidative stress is a biomarker of effects in studies assessing the health risks of occupational chemicals that can lead to mutation which can be reflected in more severe biological consequences such as genetic and neurodegenerative diseases and cancer (Tohgi et al., 1999; Schwemmer et al., 2000). Exogenous and endogenous oxidants frequently cause oxidative damage to DNA (Halliwell, 1999; Hirabayashi et al., 1999). Various markers of oxidative stress have been used to assess effects of chemicals (Abu-Qare and Abou-Donia, 2001a). Among these are an increase in the levels (8-OHdG) and an induction of 3-nitrotyrosine (Salman-Tabchen et al., 1995; Kasai, 1997; Helbock et al., 1999; Hensley et al., 1998; Maruyama et al., 1996; Oats et al., 1999; Shen et al., 1999; Takeuchi et al., 1999; Pennathur et al., 1999; Abu-Qare and Abou-Donia, 2000; Abu-Qare et al., 2001). The levels of these biomarkers were determined in biological fluids following exposure to chemicals (Cadet et al., 1998; Evans et al., 1999; Frost et al., 2000).

Veterans are likely to be exposed to both PB and sarin. Results from our laboratory showed that combined exposure to PB with DEET and permethrin could cause oxidative damage (Abu-Qare and Abou-Donia, 2000; Abu-Qare et al., 2001). Possible oxidative stress following concurrent exposure to PB and sarin has not been examined. In this study, results of analysis of urine levels of 3-nitrotyrosine, and 8-hydroxy-2'-deoxyguanosine (8-OHdG) as biomarkers of oxidative stress following administration of sarin and PB, alone and in combination in rats are presented.

2. Materials and methods

2.1. Chemicals and materials

Sarin (*O*-isopropylmethylphosphonofluoridate)

was obtained from the U.S. Army Medical Research and Material Command, (Fort Detrick, MD). Pyridostigmine bromide (PB; 3-dimethylaminocarbonyloxy-*N*-methyl pyridinium bromide), tyrosine, 3-nitrotyrosine, 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 2'-deoxyguanosine (2-dG) were purchased from Sigma Chemical Co. (St. Louis, MO). Water (HPLC grade) and acetonitrile were obtained from Mallinckrodt Baker, Inc. (Paris, Kentucky, USA). C₁₈ Sep-Pak^R cartridges were obtained from Waters Corporation (Waters Corporation, Milford, MA).

2.2. Experimental animals

Sprague Dawley rats (200–240 g) were purchased from Zivic Miller (Zelienople, PA). The untreated animals were kept in a 12 h light/dark cycle (temperature 21–23 °C) and were provided with a free supply of feed (Rodent Laboratory Chow, Ralston Purine Co., St. Louis, MO) and tap water. Animal care was conducted according to institutional guidelines.

2.3. Animal treatment and handling

Five rats were treated with a single intramuscular dose of 80 µg/kg of sarin or a single oral dose of 13 mg/kg of PB (Veterans are usually receive PB in the form of tablets), or with their combination (Abu-Qare and Abou-Donia, 2001b; Abu-Qare et al., 2001). PB dose was estimated by the US Department of Defense based on calculation of real-life exposure. One group of rats was administered both PB and sarin. Five untreated control rats were treated with oral and intramuscular dose of normal saline. The animals were kept in metabolic cages to allow for collection of urine samples. Urine samples were collected before the treatment and considered as blank samples. They were used in validation the analytical method (recovery). Urine samples were collected from the control and treated animals at the same time. The samples were collected after 16, 24, 48, 72, and 96 h following dosing and stored at –20 °C prior to analysis.

2.4. Calibration curves, limits of detection and recovery

Standard calibration curves of a concentration between 0.1–10 µg/ml of tyrosine, 3-nitrotyrosine, 2-dG, and 8-OHdG were determined under the described HPLC conditions. Detection limits were determined as the lowest concentration that can be detected taking into consideration a 1:3 baseline: peak signal ratio. Recoveries of the chemicals from urine samples were determined for concentrations of 0.1–10 µg/ml. A known concentration was spiked with control urine samples and analyzed as described under sample preparation. Amounts of tyrosine, 3-nitrotyrosine, 2-dG, and 8-OHdG were corrected based on the recovery obtained.

2.5. Sample preparation

A volume of 2 ml of the urine samples was acidified (pH 5) using 0.1 N acetic acid, then applied on a disposable C₁₈ Sep-Pak Vac 3 ml (500 mg) cartridges (Waters Corporation, Milford, MA) previously conditioned with 2 ml of methanol, and equilibrated using 2 ml of water and 2 ml of 0.03 M potassium phosphate dibasic buffer (pH 5) prior to use. After washing with 2 ml of potassium phosphate dibasic buffer (pH 5) and 2 ml of water, the sample was eluted using 2 × 1 ml of methanol, the methanolic eluates were reduced to 500 µl using stream of nitrogen, prior to analysis by HPLC.

2.6. Analysis

A volume of 10 µl of the extracts was injected into the HPLC system as described above. The mobile phase consisted of 86% water (adjusted to pH 3 using 0.1 M acetic acid), and 14% methanol at flow rate of 0.5 ml/min. The eluents were monitored by UV detection at 274 nm for tyrosine and 3-nitrotyrosine, and at 254 nm for 2'-dG and 8-OHdG. The chromatographic analysis was performed at ambient temperature. Amounts of tyrosine, 3-nitrotyrosine, 2'-dG, and 8-OHdG were calculated and corrected for the urine volume and total body weight of the animal. Con-

centrations of 2-dG and 8-OHdG or tyrosine and 3-nitrotyrosine were first calculated. The amount calculated per ml of urine excreted at that time and based on body weight of the animal and then the ratio was obtained and presented in the results. Identity of the chromatographic peaks was confirmed by spiking the urine samples with standard solutions and monitor the peaks after re-injection the samples.

2.7. Statistical analysis

Ten samples were used for the analysis at each time point. This represent tow replicates for each animal (5 animals). Analysis of Variance (ANOVA) using a GraphPad Prism program for Windows (GraphPad Software, Inc., San Diego, CA) was used to determine if the difference between treated and control samples is significant. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Clinical observation

Neither a single dose of PB or sarin produced adverse clinical effects e.g. body weight, consumption of food or water or observable toxic symptoms. There was no significant difference of urine volume between treated and control animals Administration of a combined dose of PB and sarin caused severe tremors, seizure, and convulsion.

3.2. Calibration curves, detection limits and recovery

Calibration standard curves for tyrosine, 3-nitrotyrosine, 2-dG, and 8-OHdG were obtained for a concentration range between 1–10 ng (100–1000 ng/ml). Recovery of the above chemicals from control urine samples was determined for concentrations ranged between 1–10 ng (100–1000 ng/ml). Average percentage average recoveries were $84.8 \pm 9.2\%$, $75.5 \pm 6.8\%$, $78.4 \pm 9.2\%$, $76.2 \pm 6.3\%$ for tyrosine, 3-nitrotyrosine, 2-dG, and (8-OHdG), respectively. Limits of detection of

tyrosine, 3-nitrotyrosine, 2-dG, and 8-OHdG were 0.2 ng, 0.2 ng, 0.5 ng, and 0.5 ng for tyrosine, 3-nitrotyrosine, 2-dG, and 8-OHdG, were 0.2 ng, 0.2 ng, 0.5 ng and 0.5 ng respectively.

3.3. HPLC analysis

Fig. 1 shows the chromatogram of urine samples for tyrosine, 3-nitrotyrosine, 2-dG, and 8-OHdG under described HPLC conditions. Retention times were 8.8, 9.2, 11.8, and 13.7 min 2-dG, tyrosine, 8-OHdG, and 3-nitrotyrosine, respectively.

3.4. Levels of 2-deoxyguanosine and 8-hydroxy-2-deoxyguanosine

A single oral dose of 13 mg/kg of PB and a single intramuscular dose of 80 µg/kg of sarin did not cause significant effects on urinary excretion of 8-OHdG (Fig. 2). A combination of PB and sarin significantly increased urinary excretion of 8-OHdG starting 48 h after dosing. Amount of 8-OHdG and 2'-dG was corrected according to total volume of the urine and body weight of the animal. Levels of 8-OHdG and 2-dG were calculated based on the percentage recovery of each chemical and then the ratio of 8-OHdG to 2-dG

was calculated. There was no significant difference in the amount of urine (ml) excreted between treated and control animals.

3.5. Amounts of tyrosine and 3-nitrotyrosine

Concentrations of tyrosine and 3-nitrotyrosine were corrected according to total volume of the urine and body weight of the animal and based on the percentage recovery of each chemical. A combined dose of PB and sarin caused significant increase in the urinary levels of 3-nitrotyrosine 48 h after the treatment (Fig. 3). A single dose of PB or sarin, alone did not produce statistically significant effect on level of urine 3-nitrotyrosine.

4. Discussion

The results of this study indicate that concurrent administration of sarin and PB produced significant increases in the concentrations of 8-OHdG and 3-nitrotyrosine in urine that collected 24 h after dosing, suggesting oxidative stress. In this study a real-life exposure levels of both compounds as determined by US Department of Defense (Personal Communications) was used. The elevation of urinary excretion of 8-OHdG in this experiment is consistent with previous studies reporting an increase in 8-OHdG level following exposure to chemicals, e.g., combined dose of DEET (*N,N*-diethyl-*m*-toluamide) and permethrin in rats (Abu-Qare and Abou-Donia, 2000), trichloroethylene and perchloroethylene in rats (Toraason et al., 1999), safrole (Liu et al., 1999), 2-nitropropane in rats (Loft et al., 1998), and δ -aminolevulinic acid in Chinese hamster ovary (CHO) cells (Yusof et al., 1999).

In this study ratio of 8-OHdG/2'-dG following combined dose of PB and sarin was increased approximately 2-fold in urine samples 48 h after dosing. This is in agreement with previous study of Wellejus et al. (2000) who reported an increase of 8-OHdG excretion rate from 129 to 147 pmole 24 h after *iv* dose of iron in rats. The induction of 3-nitrotyrosine as reported in this study is in agreement with previous reports of Pennathur et al. (1999) who showed that 1-methyl-4-phenyl-

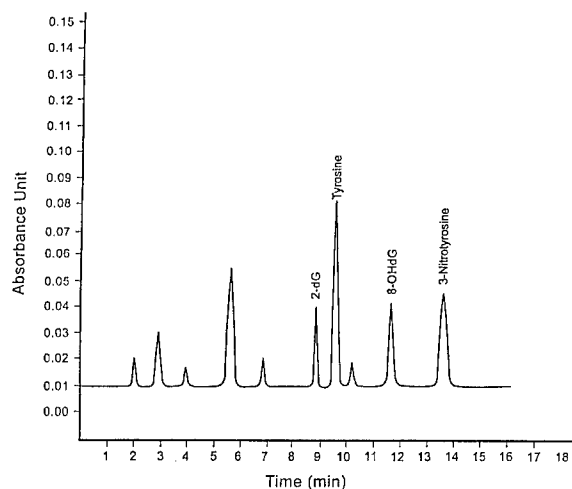


Fig. 1. HPLC Chromatogram of urine sample of 2'-dG, tyrosine, 8-OHdG, and 3-nitrotyrosine following combined dose of PB and sarin under established HPLC conditions.

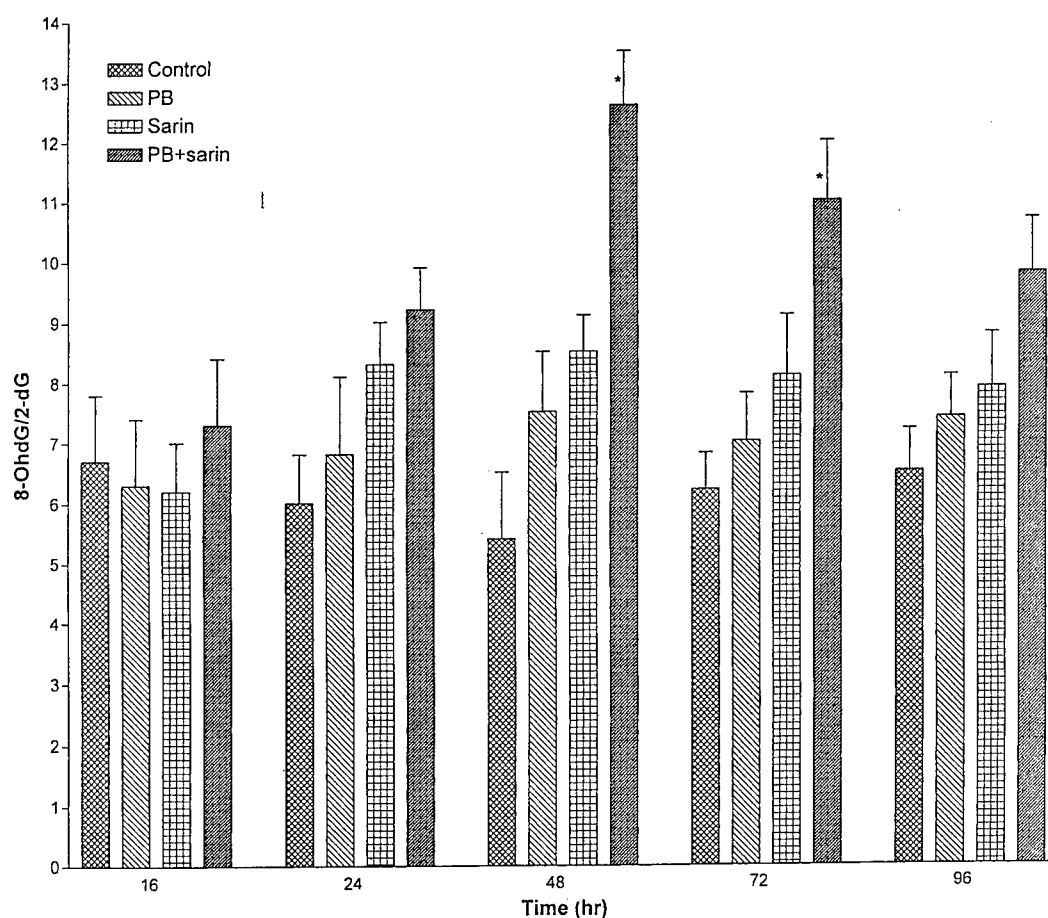


Fig. 2. Ratio of 8-OHdG to 2-dG in urine samples collected following a single oral dose of PB and a single intramuscular dose of sarin, alone and in combination. * Significantly different from controls at $P < 0.05$.

1,2,3,6-tetrahydropyridine (MPTP) increased levels of 3-nitrotyrosine in brain tissues. Furthermore, Imam et al. (1999) reported that administration of methamphetamine in mice resulted in significant formation of 3-nitrotyrosine in the striatum.

Separation of tyrosine, 3-nitrotyrosine, 2-dG, and 8-OHdG in urine samples was achieved under the described HPLC condition using UV detection at 274 nm and 254 nm, respectively. The limits of detection in this method were 0.2 ng for tyrosine, 3-nitrotyrosine and 0.5 ng for 8-OHdG and 2-dG. In previous study, limits of detection of 3-nitrotyrosine in reprefused mouse brain using HPLC with UV detection at 274 nm was 0.6 μ M

(Hirabayashi et al., 1999) and 0.1 pmole in human plasma using HPLC method. Furthermore, Yi et al. (2000) reported a limit of detection for 3-nitrotyrosine from biological tissues and fluids of 1 pg/ μ l using HPLC-MS method. The ability to detect 3-nitrotyrosine and 8-OHdG in the samples analyzed (2 ml urine) proved that our method is adequate in measuring levels of 3-nitrotyrosine and 8-OHdG in urine as biomarkers of oxidative stress.

Formation of 8-OHdG and 3-nitrotyrosine is believed to be through radical generating mechanism. Pannala et al. (1998) reported that hydroxycinnamates antioxidants decreased peroxynitrite-mediated nitration of tyrosine in

vitro. Cabassi et al. (2000) reported that the antioxidants *N*-acetyl-*L*-cysteine and melatonin reversed formation of 3-nitrotyrosine in rat heart. Furthermore, the addition of 7-nitroindazole a nitric oxide synthase inhibitor suppressed the formation of 3-nitrotyrosine to below the detection limits in the early phase of after reperfusion of mouse brain (Hirabayashi et al., 1999). Similar findings of inhibition of formation of 3-nitrotyrosine were reported following incubation of dopamine with peroxynitrite in the presence of tyrosine (Kerry and Rice-Evans, 1999). Furthermore, Niwa et al. (1999) showed that the antioxi-

dants caffeic acid and sinapinic acid inhibited formation of 3-nitrotyrosine in protein treated with peroxynitrite.

The results showed that combined administration of intramuscularly dose of sarin and an oral dose of PB increased levels of rat urinary 3-nitrotyrosine and 8-OhdG biomarkers of oxidative stress. These findings may have significant effects since PB is still in use as a prophylactic agent against nerve agents such as sarin. Exposure to sarin in war situations is a possibility. Generation of reactive oxygen species could lead to long-term adverse health effects.

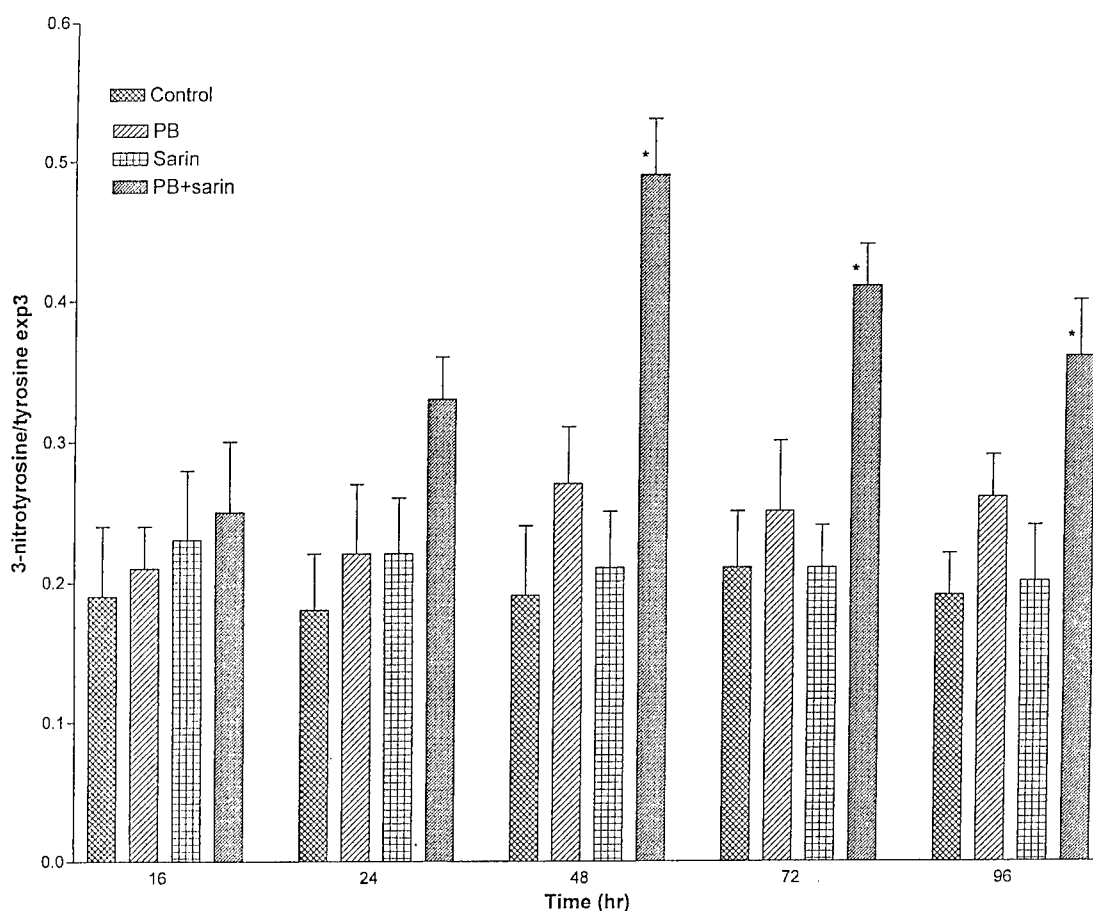


Fig. 3. Ratio of 3-nitrotyrosine to tyrosine in urine samples collected following a single oral dose of PB and a single intramuscular dose of sarin, alone and in combination. * Significantly different from controls at $P < 0.05$.

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ACUTE EXPOSURE TO SARIN INCREASES BLOOD BRAIN BARRIER PERMEABILITY AND INDUCES NEUROPATHOLOGICAL CHANGES IN THE RAT BRAIN: DOSE-RESPONSE RELATIONSHIPS

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Abstract—We hypothesize that a single exposure to an LD₅₀ dose of sarin induces widespread early neuropathological changes in the adult brain. In this study, we evaluated the early changes in the adult brain after a single exposure to different doses of sarin. Adult male rats were exposed to sarin by a single intramuscular injection at doses of 1, 0.5, 0.1 and 0.01 × LD₅₀. Twenty-four hours after the treatment, both sarin-treated and vehicle-treated (controls) animals were analyzed for: (i) plasma butyrylcholinesterase (BChE) activity; (ii) brain acetylcholinesterase (AChE) activity, (iii) m2 muscarinic acetylcholine receptor (m2 mAChR) ligand binding; (iv) blood brain barrier (BBB) permeability using [³H]hexamethonium iodide uptake assay and immunostaining for endothelial barrier antigen (EBA); and (v) histopathological changes in the brain using H&E staining, and microtubule-associated protein (MAP-2) and glial fibrillary acidic protein immunostaining. In animals treated with 1 × LD₅₀ sarin, the significant changes include a decreased plasma BChE, a decreased AChE in the cerebrum, brainstem, midbrain and the cerebellum, a decreased m2 mAChR ligand binding in the cerebrum, an increased BBB permeability in the cerebrum, brainstem, midbrain and the cerebellum associated with a decreased EBA expression, a diffuse neuronal cell death and a decreased MAP-2 expression in the cerebral cortex and the hippocampus, and degeneration of Purkinje neurons in the cerebellum. Animals treated with 0.5 × LD₅₀ sarin however exhibited only a few alterations, which include decreased plasma BChE, an increased BBB permeability in the midbrain and the brain stem but without a decrease in EBA expression, and degeneration of Purkinje neurons in the cerebellum. In contrast, animals treated with 0.1 and 0.01 × LD₅₀ did not exhibit any of the above changes. However, m2 mAChR ligand binding in the brainstem was increased after exposure to all doses of the sarin.

Collectively, the above results indicate that, the early brain damage after acute exposure to sarin is clearly dose-dependent, and that exposure to 1 × LD₅₀ sarin induces detrimental changes in many regions of the adult rat brain as early as 24 hours after the exposure. The early neuropathological changes observed after a single dose of 1 × LD₅₀ sarin could lead to a profound long-term neurodegenerative changes in many regions of the brain, and resulting behavioral abnormalities. © 2002 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: brain injury, Gulf War syndrome, glial hypertrophy, glial fibrillary acidic protein, microtubule-associated protein-2, neuron degeneration.

Since their return from the Persian Gulf War (PGW), about 30 000 veterans have had a range of unexplained ailments such as chronic fatigue, muscle and joint pain, ataxia, skin rash, headache, loss of concentration, forgetfulness, and irritability (Institute of Medicine, 1995, 1996, 2000). Several studies suggest that the illness of Gulf War veterans is the result of exposure to a psychologically stressful environment and/or a unique combina-

tion of multiple chemicals such as pyridostigmine bromide, organophosphorus insecticides, the insect repellent *N,N*-diethyl-*m*-toluamide and chemical warfare agents, particularly sarin. It is believed that the chemical warfare agent sarin was released into the atmosphere in certain regions of the Persian Gulf following the destruction of Iraqi arsenals during the war (Committee on Veterans Affairs, 1998; PAC, 1997).

The chemical sarin (methyl isopropyl phosphonofluoridate) is an organophosphorus compound that was developed for use as a chemical weapon during the World War II. Sarin was also used during the Iran–Iraq conflict in the 1980s and the Gulf War (Ivarsson et al., 1992; Defense Science Board, 1994; Committee on Banking, Housing and Urban Affairs, 1994; Brown and Brix, 1998; Institute of Medicine, 2000). The main clinical features associated with acute sarin intoxication include seizures, fasciculations, tremors and hypothermia (Taylor, 1985). Animal studies have shown that acute

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Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; BBB, blood brain barrier; BChE, butyrylcholinesterase; EBA, endothelial barrier antigen; GFAP, glial fibrillary acidic protein; mAChR, muscarinic acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; MAP-2, microtubule-associated protein-2; PGW, Persian Gulf War; VPL, ventral postero-lateral.

exposure to higher doses of sarin causes direct effects on the cholinergic system by inhibition of acetylcholinesterase (AChE) in both the peripheral nervous system (PNS) and CNS (Wood, 1951; Gupta et al., 1991; Gunderson et al., 1992). The consequent rise in acetylcholine (ACh), leads to over-stimulation of cholinergic synapses via nicotinic and muscarinic acetylcholine receptors (nAChR and mAChR; Somani, 1992; Lotti, 2000; Spencer et al., 2000). The nAChR and mAChR mediate diverse cellular responses by distinct signaling mechanisms (Wess, 1996), and excessive accumulation of ACh can lead to activation of ligand-gated ion channels. Several studies suggest that exposure to organophosphates including the sarin can cause differential regulation of both nAChRs and mAChRs (Huff et al., 1994; Katz et al., 1997). In addition to direct cholinergic effects, sarin can induce its effects through other mechanisms which may exert its effects on mAChR, reduce evoked γ -amino butyric acid (GABA) release from neurons, and alter the level of neurotransmitters in many brain regions (Dasheiff et al., 1977; Rocha et al., 1998; Chebabo et al., 1999). Thus, it is likely that alterations in both cholinergic and non-cholinergic pathways would play a key role in the neurotoxicity induced by sarin exposure.

Like soman and other lipophilic organophosphorus compounds, sarin can cross the blood brain barrier (BBB), and cause brain damage leading to neurological symptoms (Veronesi et al., 1990; Marrs, 1993; Taylor, 1996). Sarin exposure also induces progressive, long-term effects in both humans and animal models (Kadar et al., 1995; Savage et al., 1988; Yokoyama et al., 1998). A study by Kadar et al. (1995) has shown that the neuropathological changes observed after a single higher dose of sarin are progressive and the effects increase with time after the exposure, involving brain regions that are initially unaffected. In addition, it has been reported that personnel who were exposed to lower doses of the sarin exhibited persistent neurological and psychiatric abnormalities after 5–10 years of the exposure (Sidell, 1974; Duffy et al., 1979, 1980). A significant neurological dysfunction has also been observed in people exposed to sarin in Japan after 6–8 months of the exposure (Yokoyama et al., 1998). Further, long-term changes in the electroencephalogram have been observed in rhesus monkeys following exposure to a single higher dose (5 $\mu\text{g/kg}$) or repeated lower dose of sarin (1 $\mu\text{g/kg}$; Burchfiel et al., 1976; Burchfiel and Duffy, 1982). A recent study also demonstrates increased beta amplitude in marmosets after a single lower dose of the sarin (Pearce et al., 1999).

Since the symptoms reported by veterans of the PGW involved persistent abnormal regulation of functions of the CNS, it is possible that long-term effects following exposure to sarin might have been initiated as early neural damage that persisted and/or exacerbated with time after the exposure. In this study, we tested the hypothesis that a single exposure to sarin at the LD_{50} dose and fractions thereof induces widespread early neuropathological changes in the adult brain. We rigorously evaluated the early changes in the adult brain after a single

exposure to different doses of the sarin. Adult male rats were exposed to the sarin by a single intramuscular injection at a dose of 1, 0.5, 0.1 and 0.01 $\times \text{LD}_{50}$. Twenty-four hours after this treatment, both sarin-treated and vehicle-treated (controls) animals were analyzed for plasma butyrylcholinesterase (BChE) and brain AChE activity, m2 mAChR ligand binding, BBB permeability, and histopathological changes in the brain.

EXPERIMENTAL PROCEDURES

Chemicals

A stock solution of the sarin (1.9 mg/ml in saline) was obtained from the U.S. Army Medical Research and Materiel Command (Fort Derrick, MD, USA). The chemicals acetylthiocholine iodide and butyrylthiocholine iodide were obtained from the Sigma Chemical (St. Louis, MO, USA). The chemical [*N*-methyl- ^3H]hexamethonium iodide (specific activity 18 Ci/mmol) was obtained from the U.S. Army Medical Research Institute for Chemical Defense (USAMRICD, MD, USA). The chemical [^3H]AF-DX384 (2,3-dipropylamino; specific activity 106.5 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). The polyclonal antibody against glial fibrillary acidic protein (GFAP) was obtained from Dako (Carpenteria, CA, USA). The monoclonal antibodies against microtubule-associated protein-2 (MAP-2) (SMI 52) and endothelial barrier antigen (EBA) (SMI 71) were obtained from Sternberger Monoclonals (Lutherville, MD, USA). The avidin-biotin-peroxidase kits were obtained from Vector Laboratories (Burlingame, CA, USA). All other reagents were of analytical grade and were obtained from commercially available sources.

Animals and sarin treatment

Male Sprague-Dawley rats weighing 225–250 g were obtained from Zivic-Miller Laboratories (Allison Park, PA, USA). The animals were randomly assigned to control and treatment groups and housed at 21–23°C with a 12-h light/dark cycle. They were fed with Purina certified rodent chow (Ralston Purina, St. Louis, MO, USA) and tap water *ad libitum*. The rats were allowed to adjust to their environment for 1 week before the commencement of the chemical treatment. Animal care was in accordance with the Duke University institutional animal care and use committee. For sarin treatment, animals were divided into five groups. Group 1 was comprised of control animals ($n = 15$), which received a single intramuscular injection of 0.1 ml of normal saline. Animals in Group 2 ($n = 20$), Group 3 ($n = 15$), Group 4 ($n = 15$) and Group 5 ($n = 15$) were respectively treated with a single intramuscular injection of the sarin at a dose of 1, 0.5, 0.1 and 0.01 $\times \text{LD}_{50}$. Based on a series of preliminary experiments on adult rats using intramuscular injections of the sarin in our laboratory, the LD_{50} dose of the sarin was determined to be 101 $\mu\text{g/kg}$ body weight (data not shown).

Sample preparation for biochemical studies

Twenty-four hours following treatment with either saline or sarin, five animals from each group were anesthetized with pentobarbital (100 mg/kg body weight). Blood samples were collected through heart puncture using heparinized syringes. The plasma was separated by centrifugation at 17 000 $\times g$ for 5 min. The brains were dissected out and rapidly chilled to obtain the cerebrum, the midbrain, the cerebellum, and the brainstem. The entire dissection procedure was performed rapidly on ice and tissues were snap frozen using liquid nitrogen. Both plasma and dissected brain regions were stored at -80°C for enzyme studies.

AChE, BChE and mAChR binding assays

Brain (AChE) and plasma (BChE) activity were determined according to the method of Ellman et al. (1961) but modified for assay in a Molecular Devices UV Max Kinetic Microplate reader (Molecular Devices, Sunnyvale, CA, USA), as previously described (Abou-Donia et al., 1996). Protein concentration was determined by the method of Smith et al. (1985). For the assay of mAChR, tissues were homogenized in 10 mM phosphate buffer (pH=7.4) and centrifuged at $40\,000\times g$ for 10 min and the membranes were suspended in the same buffer at the protein concentration of 1.5–2.5 mg/ml, as described by Huff et al. (1994). The m2 mAChR binding was carried out by using m2-selective ligand, [3 H]AFDX 384 at room temperature for 60 min, as described by Slotkin et al. (1999).

Analysis of the BBB permeability using [3 H]hexamethonium iodide uptake assay

[3 H]hexamethonium iodide uptake assay was performed according to the methods described elsewhere (Petralli et al., 1991; Johnes et al., 2000; Abou-Donia et al., 2001). Twenty-four hours following treatment with either saline or sarin, five animals from each group were anesthetized with sodium pentobarbital (100 mg/kg) and intravenously (i.v.) injected with [3 H]hexamethonium iodide (10 μ Ci, mixed 1:1 with cold hexamethonium iodide) to give a final dose of 0.71 mg/kg body weight (1 μ Ci/kg). Ten minutes following this injection, the blood was collected from the heart with heparinized syringes and the animals were killed by decapitation. The brains were removed and placed in ice-cold normal saline. The different brain regions (cerebrum, brainstem, midbrain and cerebellum) were rapidly dissected on ice and frozen immediately using liquid nitrogen. The plasma was separated from the whole blood by centrifugation. Both plasma and brain regions were stored at -20°C until further analyses. Regions of the cerebrum, the brainstem, the midbrain and the cerebellum (50–100 mg) and 100 μ l of plasma were oxidized in a Packard 306B tissue oxidizer (Packard Instrument, Downers Grove, IL, USA), and the extracted tritium was then counted in a liquid scintillation spectrometer (Beckman Instruments, Palo Alto, CA, USA) for 5 min. Counts were recorded as d.p.m./g of tissue or 1 ml plasma. The results are presented as ratio of tissue versus plasma, which was done by dividing d.p.m. of tissue by d.p.m. of plasma. This presentation was incorporated to clearly avoid the background that resulted from blood in the brain capillaries, as the animals were not perfused prior to analysis. This is fully consistent with the presentation of data by Petralli et al., 1991.

Histopathological analysis using H&E staining

Twenty-four hours following the treatment, five animals from each group were anesthetized with sodium pentobarbital (100 mg/kg) and perfused through the heart with saline followed by 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M Tris buffer. The brains were removed carefully and post-fixed in the same fixative for 18–24 h. The tissues were then blocked and embedded in paraffin according to the standard histological techniques. Six micrometer-thick coronal sections were cut through different brain regions. From every brain, representative coronal sections ($n=5$) through the motor and somatosensory cortex, the septal hippocampus, the dorsal thalamus and the cerebellum were stained with hematoxylin and eosin (H&E) for light microscopic observation.

Immunohistochemical staining for MAP-2, EBA and GFAP

Representative brain sections through the motor and somatosensory cortex, the septal hippocampus and the dorsal thalamus from each animal were immunohistochemically stained using antibodies against MAP-2, EBA (SMI-71 and SMI-72, Sternberger Monoclonals, 1:1000 dilution; Rosenstein et al., 1992) and GFAP (Dako, 1:10000 dilution) by employing the avidin-biotin complex staining method (Hsu et al., 1981) using reagents from Vector Laboratories.

Quantitative evaluation of the number of dying neurons in different brain regions

The numerical density of dying neurons per mm^2 area of tissue in H&E-stained sections was measured for layers III and V of the motor cortex, somatosensory cortex, ventral postero-lateral (VPL) nucleus of the thalamus, granule cell layer of the dentate gyrus, pyramidal cell layer of CA1 and CA3 subfields of the hippocampus, and Purkinje cell layer of the cerebellum in lobule 2 of the cerebellar vermis. Five sections through each of the above brain regions were employed for measurements in each animal belonging to the following four groups: (a) control animals ($n=5$); (b) animals treated with $1\times\text{LD}_{50}$ ($n=5$); (c) animals treated with $0.5\times\text{LD}_{50}$ ($n=5$); (d) animals treated with $0.1\times\text{LD}_{50}$ ($n=5$); and (e) animals treated with $0.01\times\text{LD}_{50}$ ($n=5$). Measurements in sections from various groups were performed blind using experimental codes. The coding was such that animal treatments were not known during measuring; however, sections that came from the same animal were identified. All measurements were performed using a Nikon E600 microscope equipped with eyepiece grid (Southern Micro Instruments, Atlanta, GA, USA). At a magnification of $400\times$ (using $40\times$ objective lens and $10\times$ eyepieces), dying neurons encountered within a unit area of each section were counted.

The unit area selected for measurements varied for different regions of the brain, depending on the availability of the overall area for different layers using eyepiece grid at $400\times$ magnification. The area measured was 0.019 mm^2 for layer III of the motor and somatosensory cortex, 0.063 mm^2 for layer V of the motor and somatosensory cortex, 0.013 mm^2 for the dentate granule cell layer, 0.0063 mm^2 for the CA1 pyramidal cell layer, 0.013 mm^2 for the CA3 pyramidal cell layer, 0.063 mm^2 for the VPL nucleus of the thalamus, and 0.0063 mm^2 for the Purkinje cell layer of the cerebellum. The counting of dying neurons in all groups involved only those neurons that exhibited dense eosinophilic staining in both soma and proximal dendrites. The density of neurons per unit area was transformed to the numerical density per mm^2 area of the respective brain region. The mean value for each of the many brain regions (layers III and V of the motor and somatosensory cortex, granule cell layer of the dentate gyrus, CA1 and CA3 pyramidal cell layers of the hippocampus, the dorsal thalamus, and the Purkinje cell layer of the cerebellum) was calculated separately for each animal by using data from five sections before the means and standard errors were determined for the total number of animals included per group.

Morphometric analyses of MAP-2-positive and EBA-positive immunoreactivity in different brain regions

Morphometric analyses of MAP-2-positive and EBA (SMI-71)-positive immunoreactive structures in different regions were performed by using Scion Image for Windows, based on NIH Image for Macintosh (Scion Corporation, Frederick, MD, USA). For every brain region, two sections were measured in each animal. All data were collected blind to experimental codes and means were calculated for each animal individually before the means were determined for the five animals per group.

The area occupied by MAP-2-positive immunoreactive structures per unit area of tissue (0.044 mm^2 in area) was determined for layers III and V of the motor and somatosensory cortex, granule cell layer of the dentate gyrus, CA1 and CA3 pyramidal cell layers of the hippocampus and the dorsal thalamus. The area occupied by EBA-positive immunoreactive structures per unit area of tissue (0.0176 mm^2 in area) was determined in layer V of the motor and somatosensory cortex, the dentate gyrus, CA1 and CA3 subfields of the hippocampal formation, and the thalamus. For every region, the microscopic image (using $20\times$ objective lens for MAP-2 staining and $10\times$ objective lens for EBA staining) was transferred to the computer screen by focusing the appropriate area of the immunostained section with a Nikon E600 microscope equipped with a digital camera (DAGE-MTI, CCD100) (Atlanta, GA, USA) connected to an

IBM Computer. The same intensity of light in the microscope and the same parameters in the digital camera were used to digitize all the samples from different brain regions. Images in the Scion Image are two-dimensional arrays of pixels (picture elements). Pixels are represented by 8-bit unsigned integers, ranging in value from 0 to 255. Scion Image displays zero pixels as white and those with a value of 255 as black. The background and target values were set to 145 and 255, respectively, following digitization of the original gray value image in the computer screen. These values were determined by selecting the background and target areas in several sections from both control and treated animals before commencing the measurements on coded slides for statistical analysis. This scale eliminated the background staining and retained all the target (MAP-2 or EBA-immunopositive) structures in the range (145–255). The binary image of MAP-2 or EBA-positive elements was then generated by selecting a suitable threshold value (which varied from 155 to 165) to include all the MAP-2 or EBA-positive structures without any background. The final binary image was crosschecked with the original gray value image by alternating the two images on the computer screen. Finally, the image was frozen and the area occupied by the MAP-2 or EBA-positive structures in the field was measured by selecting 'Analyze particles' command of the Scion Image program. This way, the area of individual particles (i.e. MAP-2 or EBA-immunoreactive structures) in the selected field was measured, and the sum area of all particles was stored for further calculations and statistical analysis. Since spatial calibration of the image was performed in micrometers using 'Set Scale' function of the program prior to measurements, the results from area measurements were obtained in square micrometers and converted later into square millimeters.

Data analyses

Mean values between different groups of animals were compared separately for each of the above parameters using one-way analysis of variance (ANOVA) with Student's Newman-Keuls multiple comparisons post-hoc test.

RESULTS

Clinical signs

All animals injected with $1 \times \text{LD}_{50}$ of sarin developed

excessive salivation, severe tremors, seizures, and convulsions within 5–10 min, and subsequently exhibited prolonged convulsions lasting approximately 3 h. Since all animals treated with $1 \times \text{LD}_{50}$ exhibited closely comparable level of convulsions, the intensity and the duration of convulsions were not correlated with the extent of BBB permeability and the degree of neuropathology. Out of 20 rats treated with $1 \times \text{LD}_{50}$ sarin, four animals (20%) died within the first 1–3 h following sarin administration; all other animals in this group survived the end point employed in this study (i.e. 24 h after the exposure). Animals treated with 0.5, 0.1 and $0.01 \times \text{LD}_{50}$ did not exhibit signs of cholinergic toxicity with the exception of animals that received $0.5 \times \text{LD}_{50}$ dose. In this latter group, three animals out of 15 treated (20%) exhibited mild clinical signs 25–60 min after sarin treatment.

Effects on BChE activity in plasma and AChE activity and m2 mAChRs in brain regions

The BChE activity in the plasma and AChE in the cerebral cortex, the brainstem, the midbrain and the cerebellum were assayed and the results are presented in Fig. 1. Animals treated with 1 and $0.5 \times \text{LD}_{50}$ exhibited a significant inhibition in plasma BChE (30–41% of control, $P < 0.05$; Fig. 1). However, the overall extent of BChE inhibition in plasma of animals exposed to 0.01 – $0.1 \times \text{LD}_{50}$ was not significant, as the effect was highly variable between animals within these groups. A slightly higher degree of BChE inhibition in plasma of animals exposed to $0.5 \times \text{LD}_{50}$ compared to that observed following $1 \times \text{LD}_{50}$ likely reflects a variability between animals within the two groups.

Animals treated with $1 \times \text{LD}_{50}$ also exhibited a significant inhibition of AChE in all of the above brain regions (31–44% of control, $P < 0.05$; Fig. 1). Evaluation of m2 mAChRs revealed that animals treated with $1 \times \text{LD}_{50}$ exhibited a significant decrease in m2 mAChR ligand binding in the cerebral cortex (39% of control,

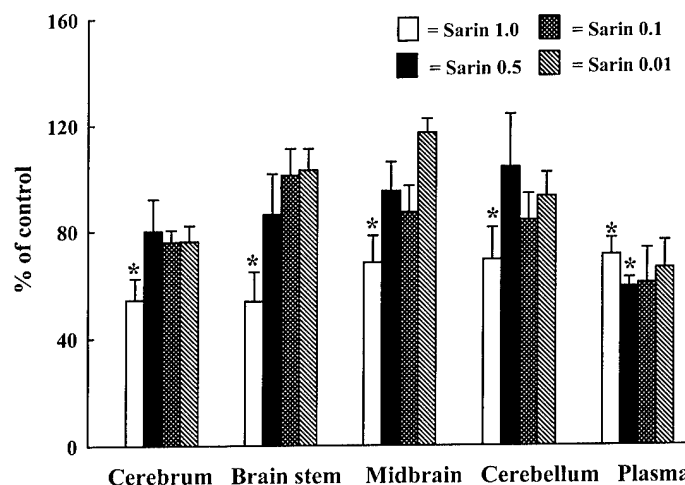


Fig. 1. The plasma BChE and brain AChE activity in different regions of the rat brain (cerebrum, midbrain, brainstem and cerebellum) at 24 h following sarin exposure. Data (mean \pm S.E.M.) are presented as percentages of corresponding control values (μmol substrate hydrolyzed/min/ml of plasma or g of tissue). Control values for brain AChE are as follows: cerebrum, 70.234 ± 7.672 ; midbrain, 152.9 ± 5.324 ; brainstem, 223.4 ± 26.908 ; cerebellum, 54 ± 2.66 . The control value for plasma BChE is 0.011 ± 0.001 . ANOVA revealed significant differences between groups in all brain regions and the plasma. $*P < 0.05$.

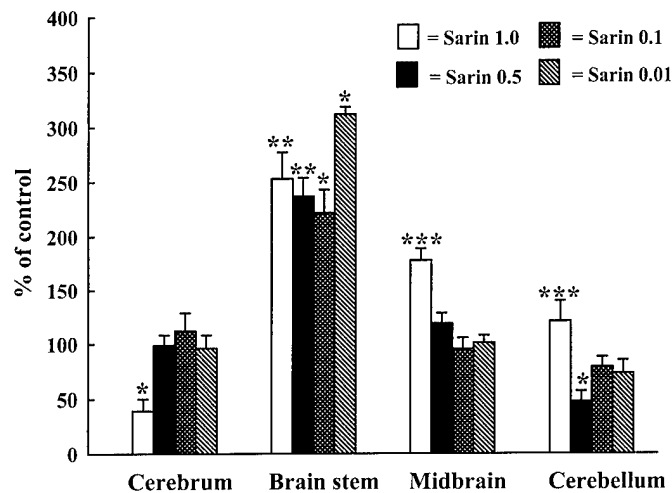


Fig. 2. The brain m2 muscarinic receptor ligand binding in different regions of the brain (cortex, midbrain, brainstem and cerebellum) at 24 h following sarin exposure. Data (mean \pm S.E.M.) are presented as percentage of corresponding controls. Control values are: cerebrum, 534.65 ± 52.36 fmol/mg of protein; midbrain, 1844.70 ± 366.82 ; brainstem, 293.374 ± 23.21 ; and cerebellum 971.388 ± 78.32 . *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

$P < 0.05$; Fig. 2); however, the receptor ligand binding was significantly increased in the brainstem (253% of control, $P < 0.01$; Fig. 2), midbrain (198.0% of control, $P < 0.001$; Fig. 2) and the cerebellum (138.0% of control, $P < 0.001$; Fig. 2). Animals treated with $0.5 \times LD_{50}$ showed a significant decrease in the cerebellum (53% of control, $P < 0.05$; Fig. 2) but, a significant increase in the brainstem (237% of control, $P < 0.01$; Fig. 2). In contrast, animals treated with either 0.1 or $0.01 \times LD_{50}$ exhibited a significant increase in only the brainstem (253% and 313% of control, $P < 0.05$; Fig. 2).

Alterations in the BBB

We evaluated the effects of a single intramuscular injection of sarin at 1.0, 0.5, 0.1, or $0.01 \times LD_{50}$ dose on BBB permeability 24 h after the treatment. The data are presented in Fig. 3. The [3H]hexamethonium iodide was used to monitor changes in the permeability

of the BBB within the cerebrum, the brainstem, the midbrain and the cerebellum. Exposure to lower doses of sarin (0.1 or $0.01 \times LD_{50}$) did not induce significant alterations in the permeability of the BBB (Fig. 3). In contrast, exposure to higher doses of sarin (1 and $0.5 \times LD_{50}$) lead to a significant increase in BBB permeability. With $1 \times LD_{50}$ exposure, BBB permeability was dramatically increased in the cerebrum, the midbrain, the brain stem and the cerebellum (144–183% of control, $P < 0.01$; Fig. 3). With $0.5 \times LD_{50}$ exposure, BBB permeability was significantly increased in only the midbrain and the brain stem (138–168% of control, $P < 0.01$; Fig. 3).

The alterations in BBB permeability were also assessed by EBA immunostaining that shows BBB protein in brain capillaries and also in smaller vessels invading the brain parenchyma (Fig. 4). Earlier studies have demonstrated that a dramatic reduction in EBA staining is indicative of an alteration in the BBB (Jensen et al.,

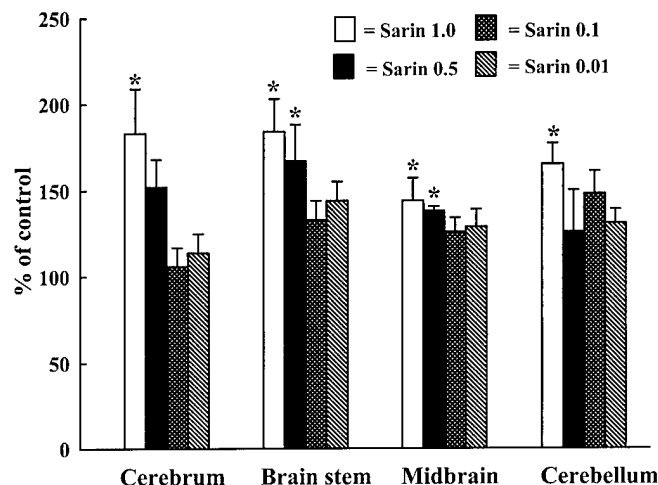


Fig. 3. [3H]Hexamethonium iodide uptake in different brain regions (cortex, midbrain, brainstem and cerebellum) at 24 h following sarin exposure. Data (mean \pm S.E.M.) are presented as the percentage of control animals of the ratio of tissue uptake (d.p.m./g) versus that of the plasma (d.p.m./ml; T/P) in control animals. Control values are as follows: cortex, 0.317 ± 0.035 ; midbrain, 0.223 ± 0.018 ; brainstem 0.224 ± 0.019 ; and cerebellum 0.173 ± 0.011 . * $P < 0.05$.

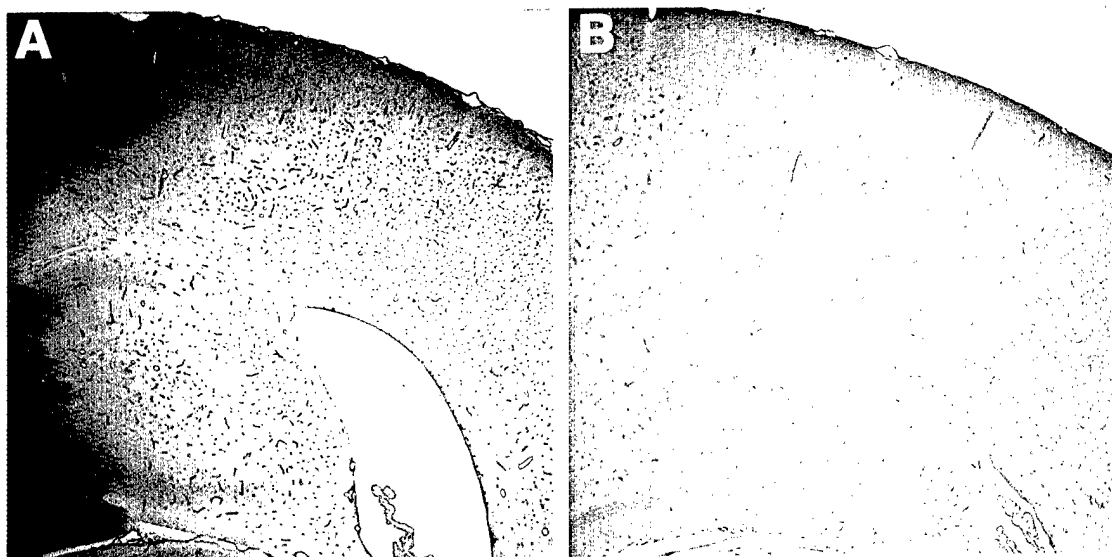


Fig. 4. Expression of EBA in the cerebral cortex and the hippocampus at 2 h following sarin exposure. (A) shows EBA staining pattern in a coronal section of brain through the cortex and the hippocampus from a control animal. (B) shows a coronal section of the brain from an animal killed 2 h after a single intramuscular injection of $1 \times LD_{50}$ of sarin. Note that EBA expression is dramatically decreased in the sarin-treated animal, suggesting that $1 \times LD_{50}$ sarin alters the BBB as early as 2 h after the exposure.

1998). Animals treated with a single intramuscular injection of $1 \times LD_{50}$, exhibited dramatically decreased EBA immunoreactive elements at 2–24 h after sarin treatment. This was particularly evident in the cerebral cortex and the hippocampus (Fig. 4). Occasionally, decreased EBA immunostaining was also observed in animals treated with $0.5 \times LD_{50}$ dose of sarin. In contrast, animals treated with either 0.1 or $0.01 \times LD_{50}$ dose of sarin did not exhibit significant changes in the EBA immunostaining compared to control animals. Quantification of the area of EBA (or SMI-71) immunoreactive elements per unit area of the motor and somatosensory cortex, the dentate gyrus, the CA1 and CA3 subfields of the hippocampus and the dorsal thalamus at 24 h following sarin treatment demonstrated that only the animals treated

with $1 \times LD_{50}$ showed a dramatic decrease in EBA immunostaining in all of the above regions at 24 h after the exposure (Fig. 5). The decrease in EBA immunoreactive structures was 49% in the motor cortex ($P < 0.05$), 61% in the somatosensory cortex ($P < 0.01$), 46% in the dentate gyrus ($P < 0.001$), 43% in the CA1 subfield ($P < 0.01$), 38% in the CA3 subfield ($P < 0.001$) and 38% in the VPL nucleus of the thalamus ($P < 0.001$; Fig. 5). In order to determine the earliest time at which BBB shows changes after the $1 \times LD_{50}$ sarin exposure, we quantified EBA staining in an additional group of animals ($n = 5$) killed at 2 h after $1 \times LD_{50}$ exposure. This showed comparable reductions to that observed at 24 h after the exposure (i.e. 56% in the motor cortex, $P < 0.05$; 43% in the somatosensory cortex, $P < 0.01$;

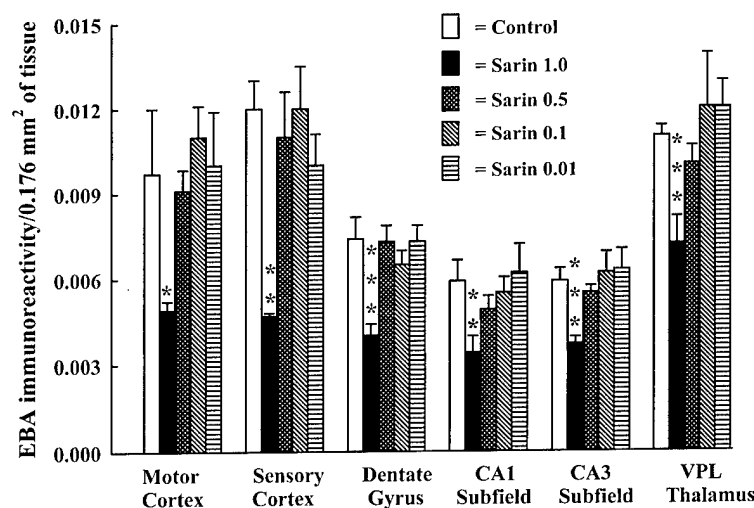


Fig. 5. Histograms show the extent of EBA immunoreactive elements, in mm^2 per unit area ($0.176 mm^2$) of the motor cortex, the sensory cortex, the dentate gyrus, CA1 and CA3 subfields, and the VPL nucleus of the thalamus. Animals were treated with a single intramuscular injection of 1, 0.5, 0.1 and $0.01 \times LD_{50}$ of sarin and analyzed for EBA staining at 24 h after the exposure. Values represent means and standard errors ($n = 5$ per group). *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

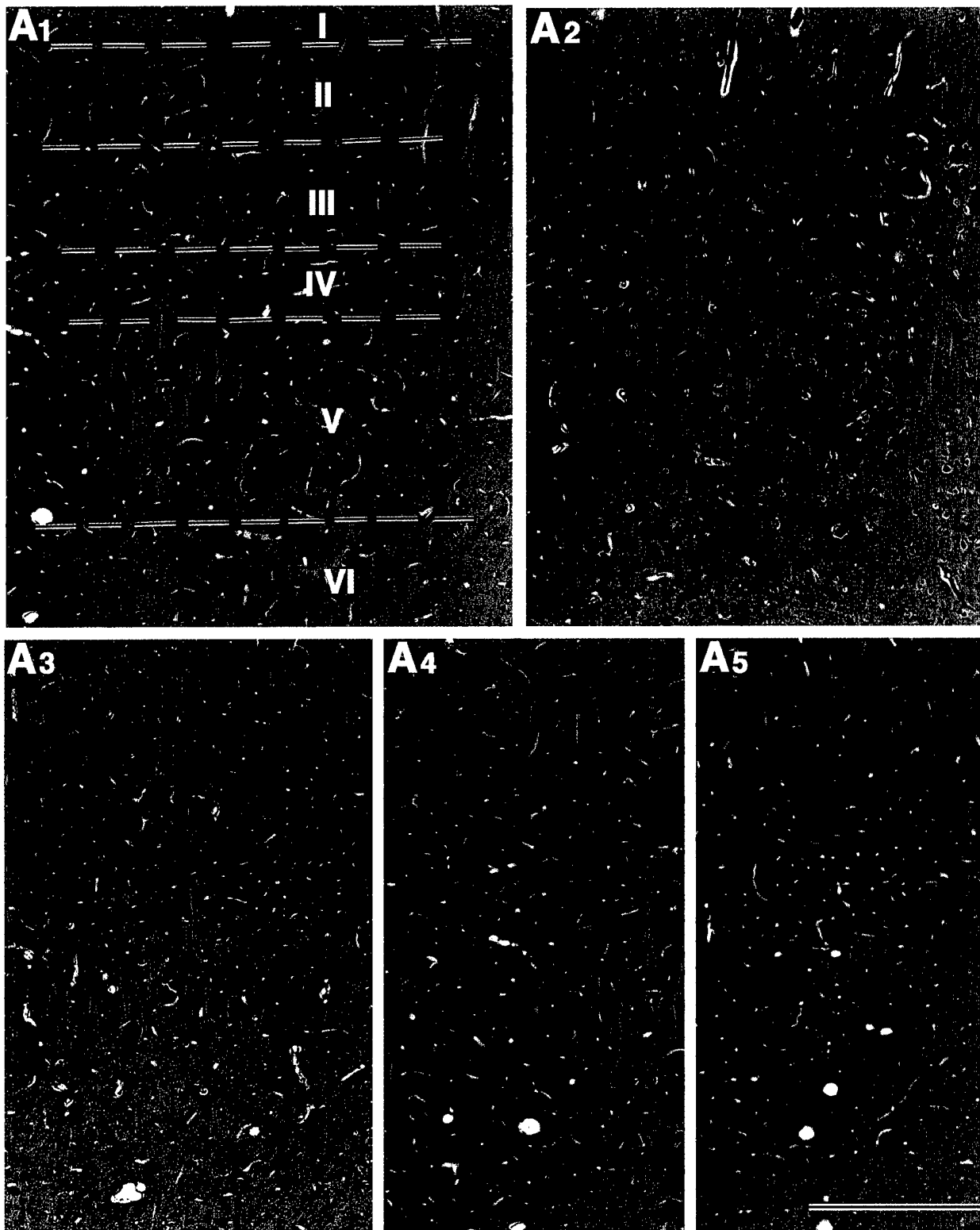


Fig. 6. Alterations in different layers (layers I–VI) of the motor cortex at 24 h following sarin exposure, visualized with H&E staining. A1 is an example from a control rat. A2 is an example from a rat that was treated with $1 \times \text{LD}_{50}$ sarin. A3 is an example from a rat treated with $0.5 \times \text{LD}_{50}$ sarin. A4 is an example from a rat treated with $0.1 \times \text{LD}_{50}$ sarin. A5 is an example from a rat treated with $0.01 \times \text{LD}_{50}$ sarin. A number of degenerating neurons are clearly visible in layers II, III and V of the cerebral cortex in rats treated with $1 \times \text{LD}_{50}$ sarin (A2). Note that animals treated with 0.5 , 0.1 and $0.01 \times \text{LD}_{50}$ did not exhibit degenerating neurons. Scale bar = 200 μm .

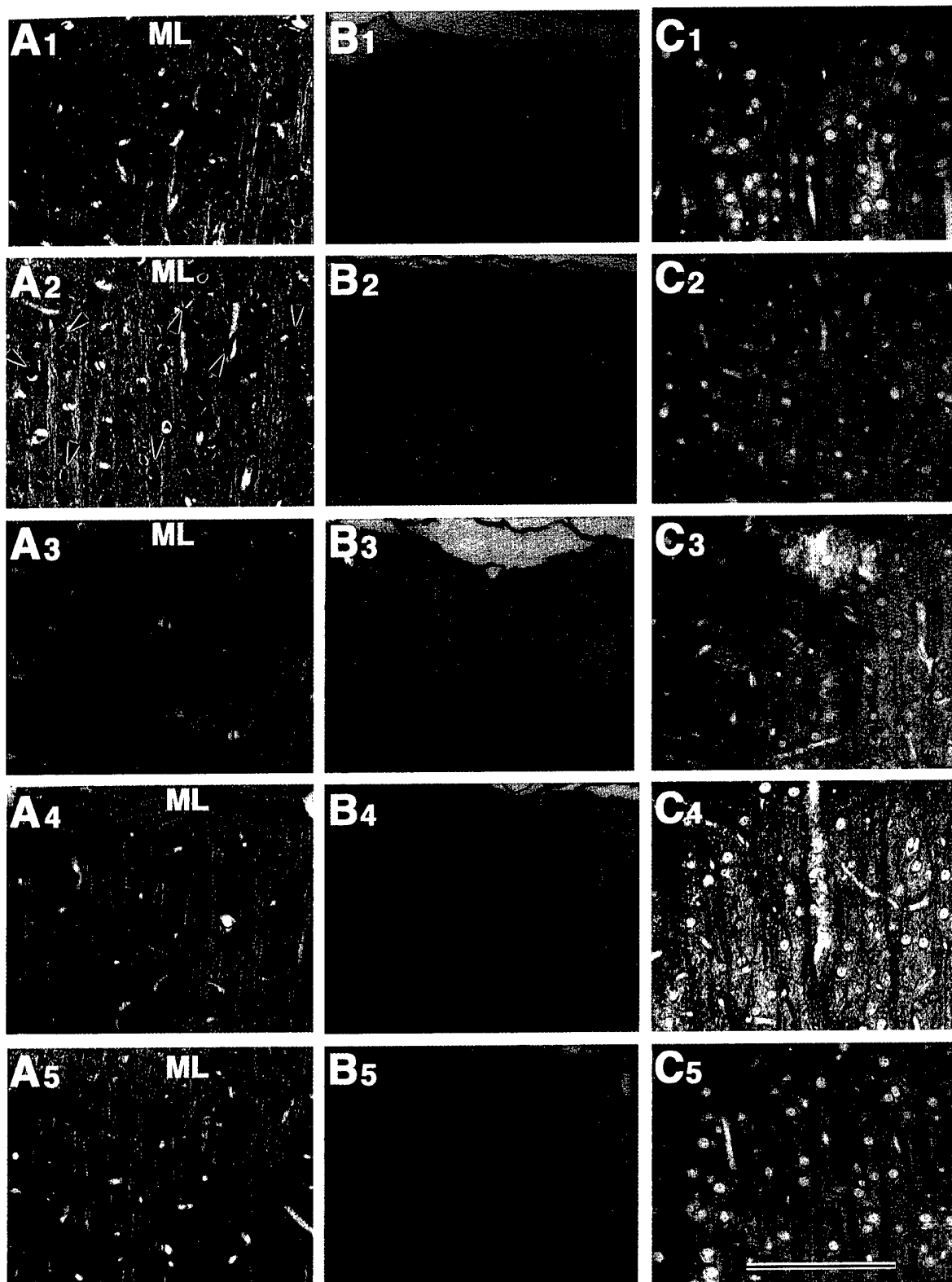


Fig. 7. Alterations in the superficial layers (layers I-III) of the motor cortex at 24 h following sarin exposure. A1-A5, H&E staining; B1-B5, EBA immunostaining; C1-C5, MAP-2 immunostaining. A1, B1 and C1 are examples from a control rat. A2, B2 and C2 are examples from a rat that was treated with $1 \times LD_{50}$ sarin. A3, B3 and C3 are examples from a rat treated with $0.5 \times LD_{50}$ sarin. A4, B4 and C4 are examples from a rat treated with $0.1 \times LD_{50}$ sarin. A5, B5 and C5 are examples from a rat treated with $0.01 \times LD_{50}$ sarin. A number of degenerating neurons are clearly visible in rats treated with sarin only at $1 \times LD_{50}$ (arrows in A2). A dramatic reduction in EBA immunostaining is also obvious in rats treated with sarin at $1 \times LD_{50}$. In addition, animals treated with $1 \times LD_{50}$ sarin exhibit significantly reduced MAP-2-positive structures with characteristically wavy appearance of dendrites. In contrast, animals treated with $0.5 \times LD_{50}$ exhibit only a slight reduction in MAP-2 immunoreactivity, and animals treated with 0.1 and $0.01 \times LD_{50}$ do not exhibit any of the above changes. Scale bar = 100 μ m.

32% in the dentate gyrus, $P < 0.001$; 51% in the CA1 subfield, $P < 0.01$; 29% in the CA3 subfield, $P < 0.001$; and 36% in the dorsal thalamus, $P < 0.001$). Thus, reductions in EBA expression occurs as early as 2 h after $1 \times \text{LD}_{50}$ sarin exposure and persists at the same level at least until 24 h after the exposure.

Additionally, we quantified the area of EBA immunoreactive elements per unit area of the midbrain and the brain stem following 1.0 and $0.5 \times \text{LD}_{50}$ sarin, as [^3H]hexamethonium iodide uptake assay suggested an increased BBB permeability in these regions. This analysis revealed a significant change in EBA staining pattern following exposure to a dose of $1 \times \text{LD}_{50}$ in comparison to controls within both brainstem (mean \pm S.E.M.: controls, 8182 ± 568 ; $1 \times \text{LD}_{50}$ sarin, 5013 ± 1148 ; $P < 0.05$) and midbrain (controls, 10648 ± 449 ; $1 \times \text{LD}_{50}$ sarin, 8765 ± 313 ; $P < 0.05$). However, with a dose of $0.5 \times \text{LD}_{50}$ sarin, the area of EBA immunoreactive elements per unit area remained similar to control values in both brainstem (8084 ± 656 , $P > 0.05$) and midbrain (9738 ± 383 , $P > 0.05$). Thus, animals intoxicated with $0.5 \times \text{LD}_{50}$ sarin show an increased BBB permeability in only brainstem and midbrain with [^3H]hexamethonium uptake assay but not with EBA analysis, suggesting only a minor or transient leakage of the BBB in these regions with $0.5 \times \text{LD}_{50}$ dose.

Histopathological changes in the brain

Evaluation of brain sections stained with H&E clearly revealed significant neuronal degeneration in rats treated with $1 \times \text{LD}_{50}$ and occasional neurodegeneration in animals treated with $0.5 \times \text{LD}_{50}$, in comparison to animals treated with 0.1 and $0.01 \times \text{LD}_{50}$, or vehicle alone. Degenerating neurons were characterized by eosinophilic staining of both cell body and proximal dendrites. In contrast, the healthy neurons in the same section exhibited hematoxylin-stained nuclei with a lightly eosin-stained perinuclear cytoplasm. The brain regions where neuronal degeneration was most obvious include the motor and somatosensory areas of the cerebral cortex, the dorsal thalamus, dentate gyrus, the CA1 and CA3 subfields of the hippocampus and the cerebellum. Other areas of the brain showed only occasional dying neurons in some animals.

Alterations in the cerebral cortex

In animals treated with $1 \times \text{LD}_{50}$, both superficial and deeper layers of the motor and somatosensory cortex exhibited degenerating neurons in H&E-stained sections. In the superficial layer (layers I–III; Figs. 6 and 7), degenerating neurons were conspicuous in all the layers. The majority of degenerating neurons in these layers were of the pyramidal type with prominent eosinophilic apical dendrites (Figs. 6 and 7). In deeper layers of the cortex (layers IV and V), degenerating neurons were mostly observed in the layer V (Figs. 6 and 8). These are larger pyramidal neurons with prominent apical and basal dendrites emanating from a larger pyramidal-shaped cell body (Fig. 8). Only a few degenerating

neurons were observed in animals treated with $0.5 \times \text{LD}_{50}$ in both superficial and deeper layers of the motor and somatosensory cortex (layers II and V, Figs. 6, 7 and 8). Further, the overall cytoarchitecture of the cortex of animals treated with $0.5 \times \text{LD}_{50}$ was comparable to animals treated with 0.1 and $0.01 \times \text{LD}_{50}$ and vehicle-treated control animals (Figs. 6, 7 and 8). The adjacent sections stained for MAP-2 substantiated the above finding in animals treated with $1 \times \text{LD}_{50}$ dose by exhibiting a greatly reduced MAP-2 staining of dendrites in both superficial and deeper layers of the cortex (Figs. 7 and 8). Animals treated with $0.5 \times \text{LD}_{50}$ also showed a slight reduction in MAP-2 immunostaining (Figs. 7 and 8). MAP-2 immunostaining in animals treated both 0.1 and $0.01 \times \text{LD}_{50}$ or vehicle showed normal distribution of MAP-2-positive dendrites and neuronal perikarya within both superficial and deeper layers of the cerebral cortex (Figs. 7 and 8). Evaluation of GFAP immunoreactivity at 24 h after the exposure showed no changes in the different treated groups, in comparison to the control animals (data not illustrated).

Extent of neuron loss and reductions in MAP-2 immunoreactivity within the motor and somatosensory cortex and the thalamus

Quantification of dying neurons per mm^2 area of the motor and somatosensory cortex and the VPL nucleus of the thalamus revealed significant neuronal cell death in all of the above regions in animals treated with $1 \times \text{LD}_{50}$ of sarin ($P < 0.001$; Fig. 9) in comparison to control animals. Further comparison revealed that treatment with 0.5, 0.1, or $0.01 \times \text{LD}_{50}$ of sarin does not result in significant neuronal cell death at 24 h after the exposure (Fig. 9). Thus, acute exposure to a larger dose of sarin ($1 \times \text{LD}_{50}$) leads to a significant early neuronal cell death in the motor and somatosensory cortex, and the dorsal thalamus. Quantification of the area of MAP-2-immunoreactive elements per unit area of the motor cortex, the somatosensory cortex and the VPL nucleus of the thalamus at 24 h following sarin treatment (1, 0.5, 0.1 and $0.01 \times \text{LD}_{50}$) demonstrated the same trend observed for neuronal cell death. The only exception is the VPL nucleus of the thalamus, which exhibited no significant differences at all doses in comparison to the control animals. Animals treated with 0.5, 0.1 and $0.01 \times \text{LD}_{50}$ of sarin did not exhibit a significant reduction in MAP-2-positive structures in all of the above regions (Fig. 9). However, animals treated with $1 \times \text{LD}_{50}$ exhibited 44% reduction in MAP-2-positive elements in the motor cortex ($P > 0.05$) and 34% reduction in the somatosensory cortex ($P > 0.05$; Fig. 9).

Alterations in the hippocampal formation

Neuronal degeneration was obvious in the dentate gyrus, and the CA1 and CA3 subfields of the hippocampal formation following exposure to sarin at a dose of $1 \times \text{LD}_{50}$ (Figs. 10–13). In the dentate gyrus, degenerating neurons were observed in both the granule cell layer and the dentate hilus (Figs. 10 and 11). However, MAP-2

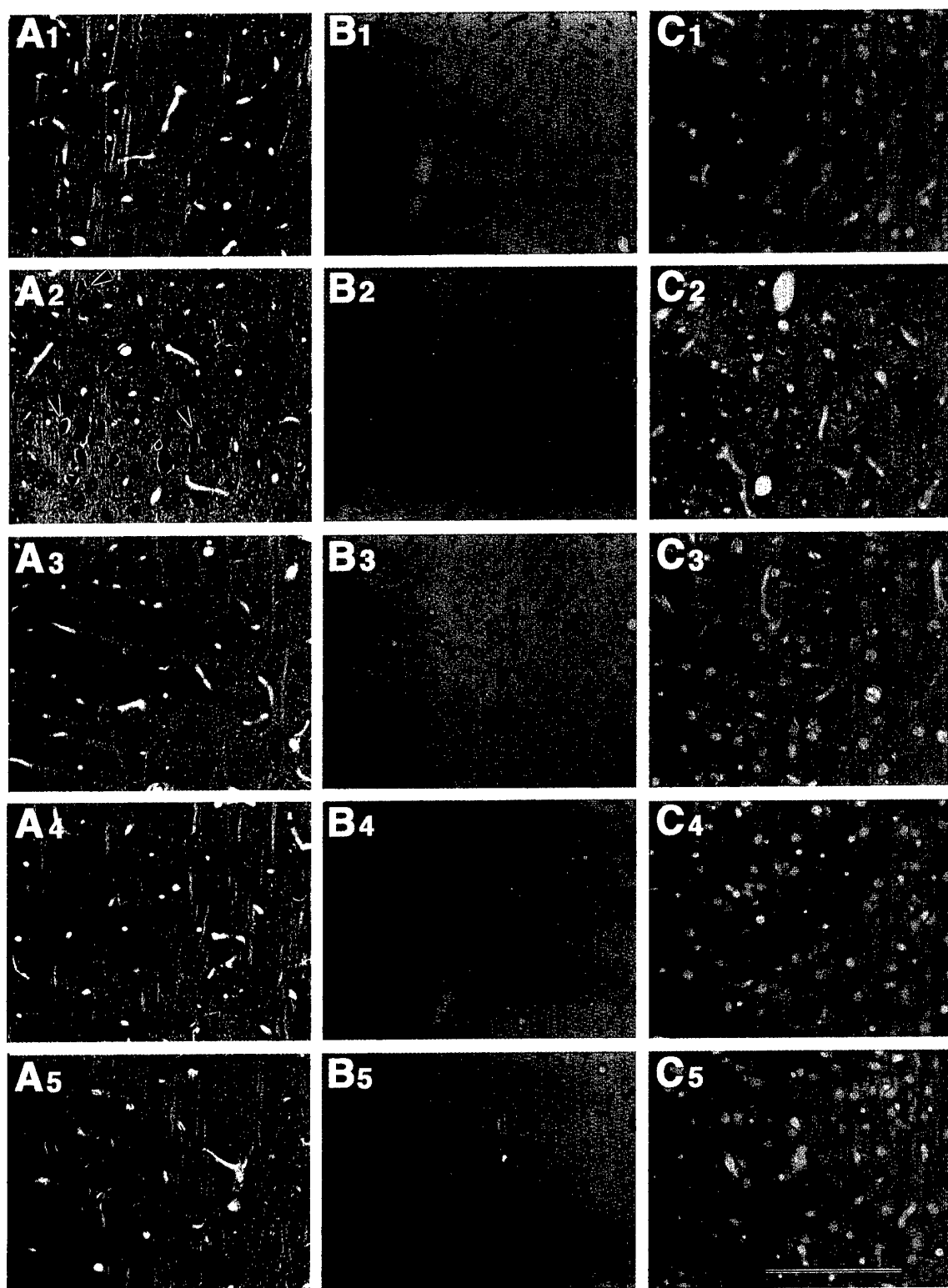


Fig. 8. Changes in the deeper layers (layers IV and V) of the motor cortex at 24 h following sarin exposure. A1–A5, H&E staining; B1–B5, EBA immunostaining; C1–C5, MAP-2 immunostaining. A1, B1 and C1 are examples from a control rat. A2, B2 and C2 are examples from a rat that was treated with $1 \times LD_{50}$ sarin. A3, B3 and C3 are examples from a rat treated with $0.5 \times LD_{50}$ sarin. A4, B4 and C4 are examples from a rat treated with $0.1 \times LD_{50}$ sarin. A5, B5 and C5 are examples from a rat treated with $0.01 \times LD_{50}$ sarin. A number of degenerating neurons are clearly visible in rats treated with sarin at $1 \times LD_{50}$ (arrows in A2). The latter group also exhibits a dramatic reduction in EBA (B2) and MAP-2 immunoreactivity (C2); in addition, dendrites appear thicker, swollen and fragmented. However, animals treated with $0.5 \times LD_{50}$ exhibit only a slight reduction in MAP-2 immunoreactivity (C3), and animals treated with 0.1 and $0.01 \times LD_{50}$ did not exhibit any changes.

Scale bar = 100 μ m.

immunostaining of the granule cell layer and the molecular layer did not show any significant differences between animals belonging to different treated groups. In the CA1 subfield of the hippocampus, degenerating neurons were clearly observed in the stratum pyramidale of animals treated with $1 \times LD_{50}$ of sarin (Figs. 10 and 12). Evaluation of MAP-2 immunostaining showed greatly decreased expression of MAP-2-positive dendrites in animals treated with $1 \times LD_{50}$ of sarin (Fig. 12). In the latter group, alterations in MAP-2 expression of dendrites were particularly conspicuous in the CA1 stratum radiatum; the MAP-2 dendrites appeared fragmented and thinner (Fig. 12).

In the CA3 subfield of the hippocampus, the degenerating neurons were conspicuous in the stratum pyramidale of animals treated with $1 \times LD_{50}$ (Figs. 10 and 13). The MAP-2 staining of adjacent sections demonstrated a reduced density of MAP-2-positive dendrites in animals belonging to groups that received 1.0 or $0.5 \times LD_{50}$ sarin compared to control animals (Fig. 13). Evaluation of GFAP immunoreactivity in different regions of the hippocampal formation at 24 h after the exposure showed no changes in different treatment groups in comparison to the control animals (data not illustrated).

Extent of neuron loss and reductions in MAP-2 immunoreactivity in the hippocampal formation

Quantification of dying neurons per mm^2 area of the granule cell layer of the dentate gyrus and pyramidal cell layer of subfields CA1 and CA3 (Fig. 14) demonstrated that in the dentate granule cell layer, animals treated with $1 \times LD_{50}$ of sarin exhibited a significant number of dying neurons compared to control animals ($P < 0.01$; Fig. 14). In CA1 and CA3 subfields, the same trend was observed in that only animals treated with $1 \times LD_{50}$ exhibited a significant increased in dying neurons ($P < 0.001$; Fig. 14). Quantification of the area of MAP-2-immunoreactive elements per unit area of the pyramidal cell layer of subfields CA1 and CA3 exhibited a significant reduction in MAP-2-positive structures in animals treated with $1 \times LD_{50}$ dose of sarin (53–42% reduction, respectively, $P < 0.001$; Fig. 14). The CA3 region also showed a reduced MAP-2 staining in animals receiving $0.5 \times LD_{50}$ sarin (22% reduction, $P < 0.05$).

Alterations in the cerebellum

In the cerebellum, the most conspicuous damage in animals treated with 1 and $0.5 \times LD_{50}$ of sarin was in the Purkinje cell layer (Fig. 15) compared to animals treated with 0.1 or $0.01 \times LD_{50}$ sarin or control animals. Evaluation of GFAP immunoreactivity at 24 h after the exposure showed no changes in the cerebellar white matter and the cerebellar cortex of different treatment groups in comparison to the control animals (data not shown). Quantitative analysis of Purkinje cells in lobule 2 of the cerebellar vermis showed that animals treated with sarin at 1 and $0.5 \times LD_{50}$ exhibited a significant number of dying neurons in comparison to control animals (Fig. 16; $P > 0.001$).

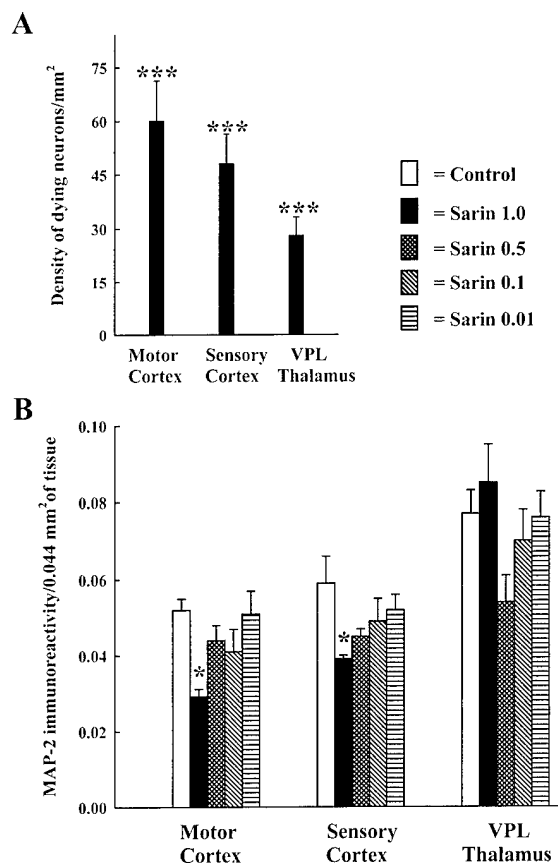


Fig. 9. Histograms show the density of dying neurons/ mm^2 (A), and MAP-2 immunoreactive elements in mm^2 (B) per unit area ($0.044 mm^2$) of the motor cortex, sensory cortex and the VPL nucleus of the thalamus in different groups. Animals were treated with a single intramuscular injection of 1, 0.5, 0.1 and $0.01 \times LD_{50}$ of sarin and analyzed at 24 h after the exposure. Values represent means and standard errors ($n = 5$ per group). *** $P < 0.001$; * $P < 0.05$.

DISCUSSION

The present study was designed to investigate the early neuropathological changes in the adult rat brain following acute exposure to different doses (1, 0.5, 0.1 and $0.01 \times LD_{50}$) of sarin. Analyses of the early effects of different doses of sarin on the brain is of considerable significance for assessing the extent of early brain damage that could occur when people are acutely exposed to either higher doses of sarin (i.e. following direct exposure during war or terrorist attacks) or lower doses of sarin (i.e. following indirect exposure). The results of this combined biochemical and neuropathological study suggest that a single exposure to sarin at $1 \times LD_{50}$ can cause extensive brain damage by 24 h mainly involving the cerebral cortex, the hippocampus (dentate gyrus and the CA1 and CA3 subfields) and the cerebellum. This was evidenced by: (i) a significant inhibition of plasma BChE, regional brain AChE, and m2 mAChR ligand binding; (ii) a dramatic increase in the BBB permeability associated with a drastic decrease in the EBA immunostaining; and (iii) a diffuse neuronal cell death coupled with decreased MAP-2 expression within dendrites of surviving neurons. Various clinical signs such as saliva-

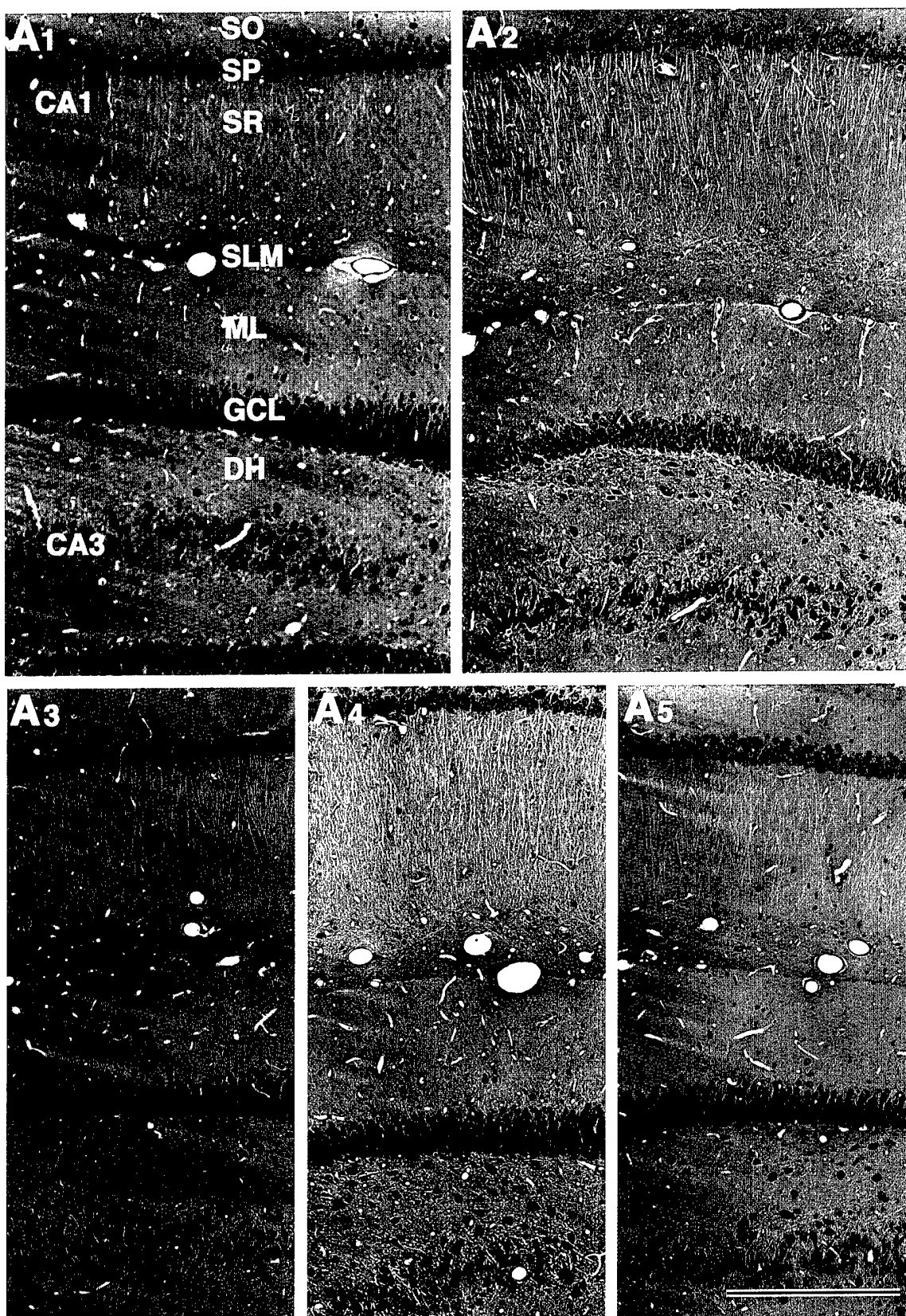


Fig. 10. Alterations in different regions of the hippocampus at 24 h following sarin exposure, visualized with H&E staining: A1 is an example from a control rat, A2 is an example from a rat that was treated with $1 \times LD_{50}$ sarin. A3 is an example from a rat treated with $0.5 \times LD_{50}$ sarin. A4 is an example from a rat treated with $0.1 \times LD_{50}$ sarin. A5 is an example from a rat treated with $0.01 \times LD_{50}$ sarin. A number of degenerating neurons are clearly visible in different regions of the hippocampus in rats treated with $1 \times LD_{50}$ sarin (A2). Note that animals treated with 0.5, 0.1 and $0.01 \times LD_{50}$ did not exhibit degenerating neurons. DH, dentate hilus; GCL, granule cell layer; SLM, stratum lacunosum moleculare; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum. Scale bar = 200 μ m.

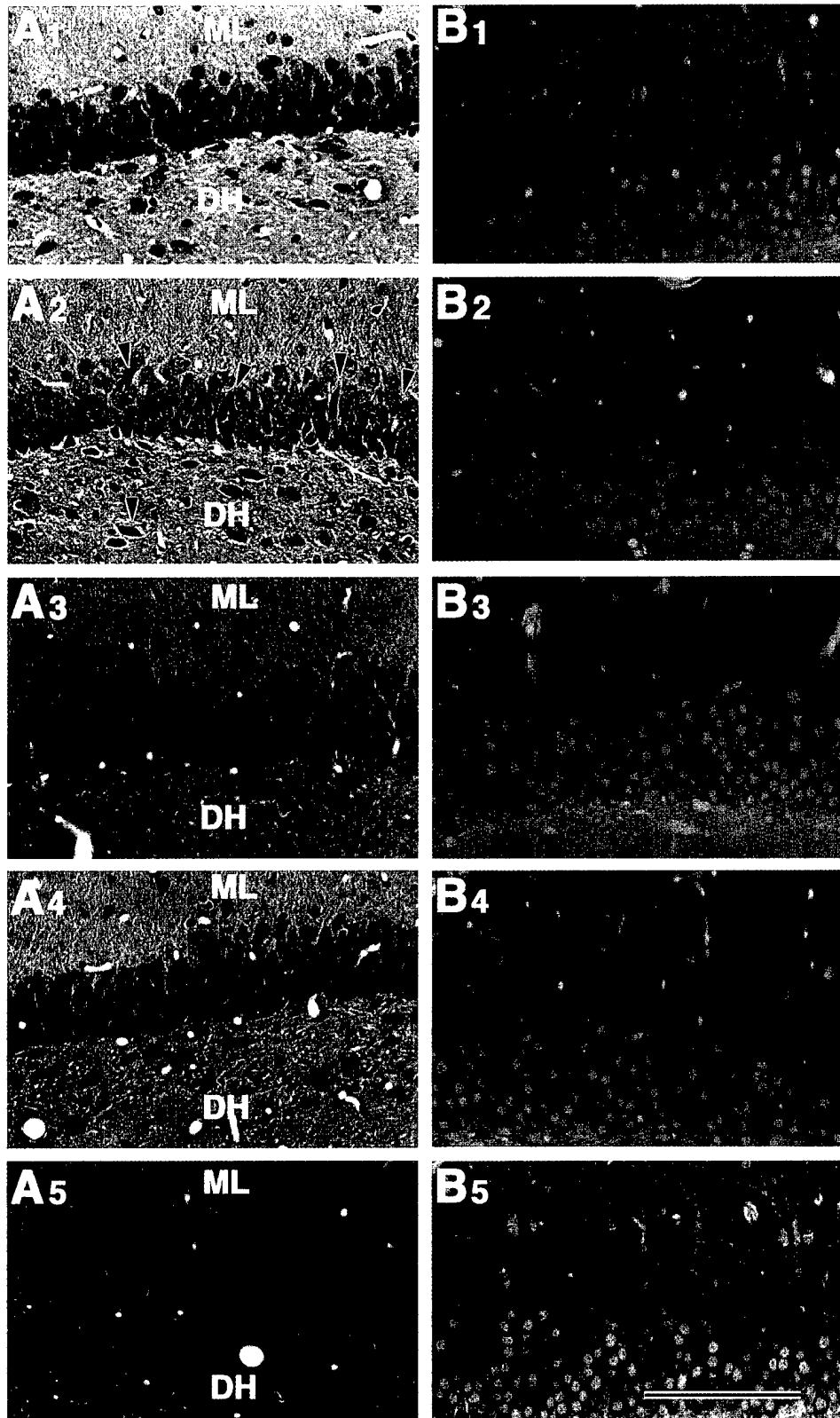


Fig. 11. Alterations in the dentate gyrus at 24 h following sarin exposure. A1–A5, H&E staining; B1–B5, MAP-2 immunostaining. A1 and B1 are examples from a control rat. A2 and B2 are examples from a rat treated with $1 \times LD_{50}$. A3 and B3 are examples from a rat treated with $0.5 \times LD_{50}$. A4 and B4 are examples from a rat treated with $0.1 \times LD_{50}$. A5 and B5 are examples from a rat treated with $0.01 \times LD_{50}$. Note a large number of degenerating neurons in the dentate granule cell layer (GCL) and the dentate hilus (DH) of rats treated with $1 \times LD_{50}$ (arrows in A2). However, MAP-2 expression in all treated animal groups appears comparable to control animals. Scale bar = 100 μ m.

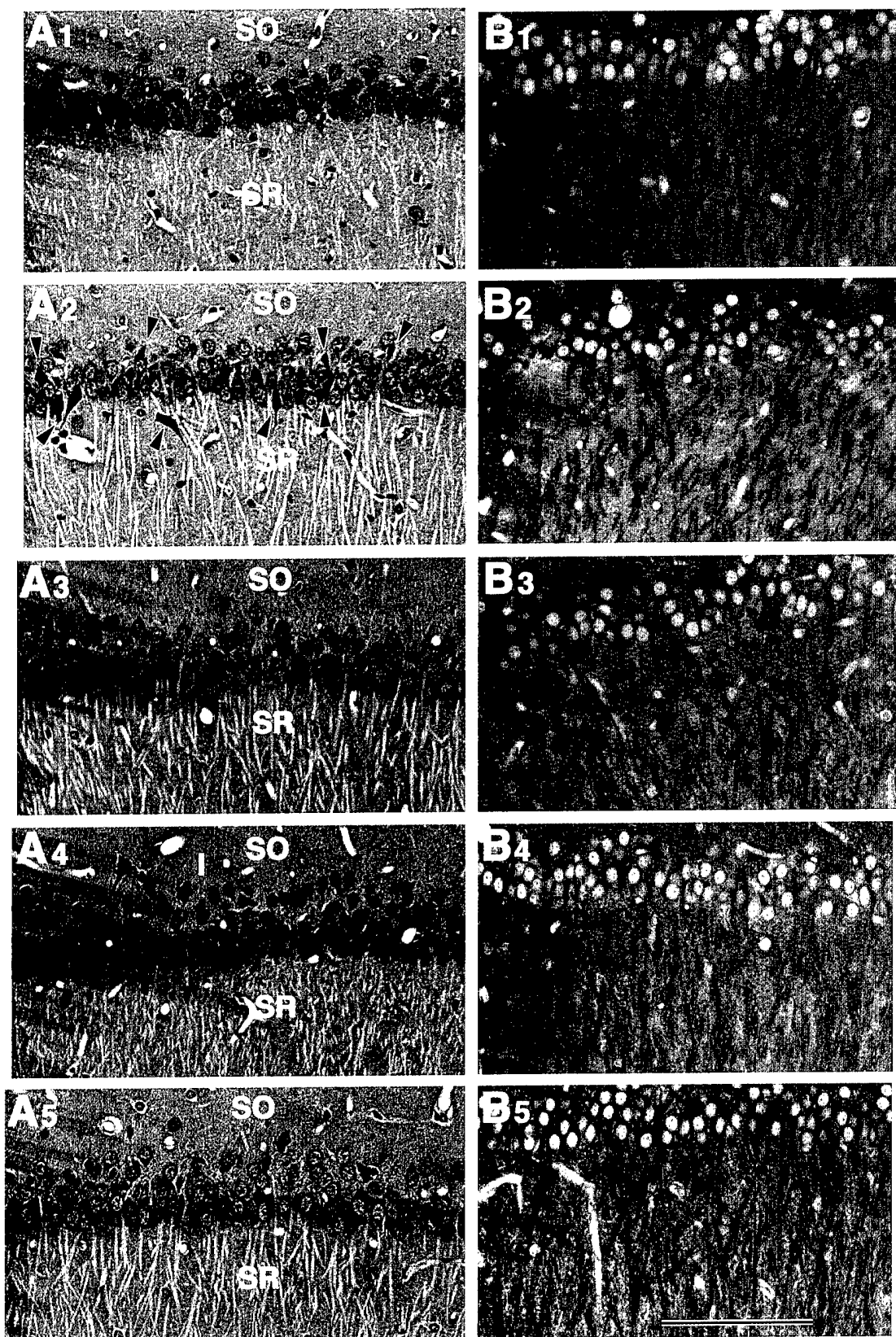


Fig. 12. Alterations in the CA1 subfield of the hippocampus at 24 h following sarin exposure. A1-A5, H&E staining; B1-B5, MAP-2 immunostaining. A1 and B1 are examples from a control rat. A2 and B2 are examples from a rat treated with $1 \times LD_{50}$ sarin. A3 and B3 are examples from a rat treated with $0.5 \times LD_{50}$. A4 and B4 are examples from a rat treated $0.1 \times LD_{50}$. A5 and B5 are examples from a rat treated $0.01 \times LD_{50}$. A large number of degenerating pyramidal neurons are clearly visible in the stratum pyramidale (SP) of rats treated with $1 \times LD_{50}$ (arrows in A2). In addition, the latter group exhibits a significantly reduced MAP-2 immunoreactivity (B2) associated with alterations in the pattern of MAP-2 expression in dendrites (B1). Scale bar, 100 μ m.

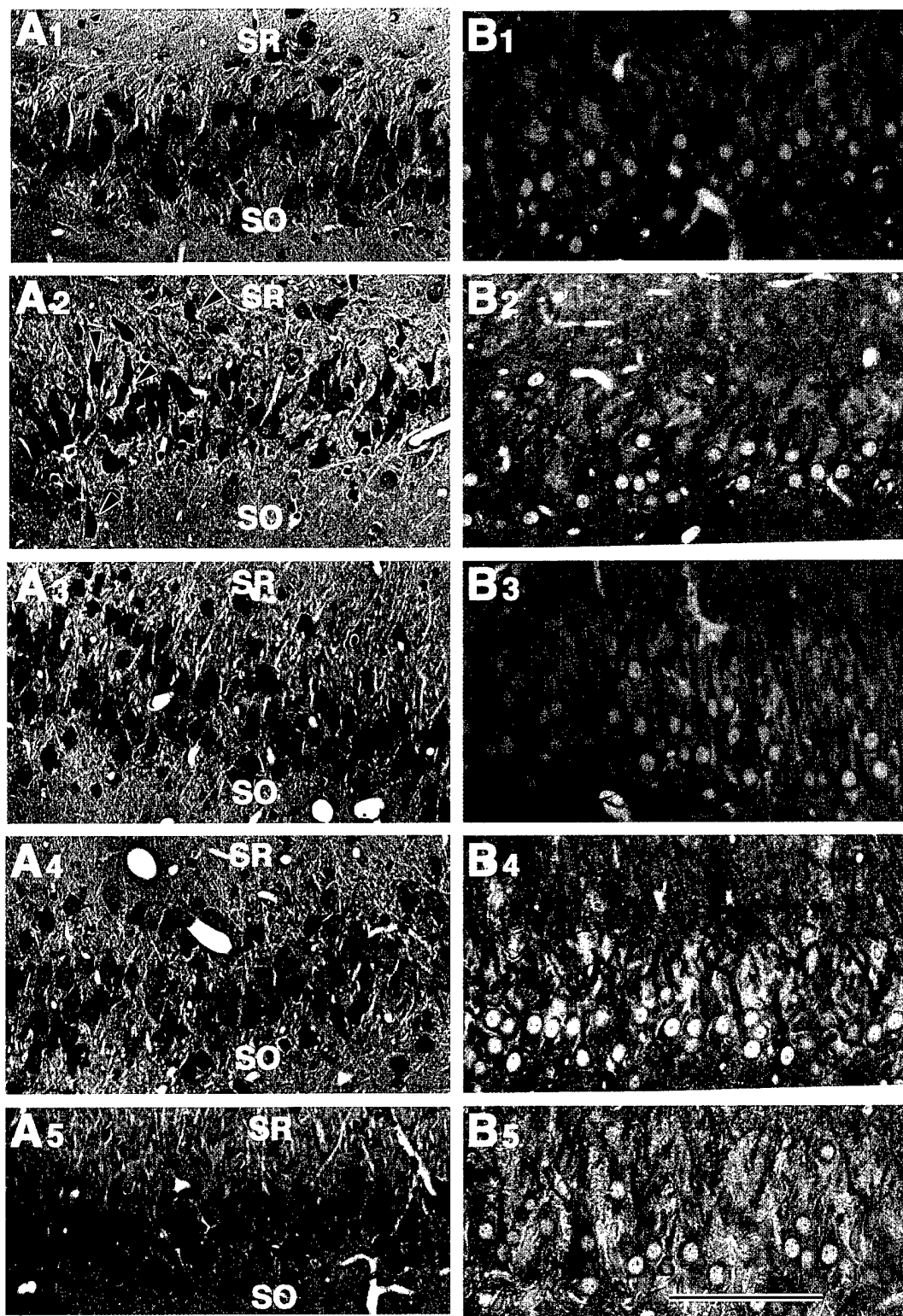


Fig. 13. Changes in the CA3 subfield of the hippocampus at 24 h following sarin exposure. A1–A5, H&E staining; B1–B5, MAP-2 immunostaining. A1 and B1 are examples from a control rat. A2 and B2 are examples from a rat treated with $1 \times LD_{50}$. A3 and B3 are examples from a rat treated with $0.5 \times LD_{50}$. A4 and B4 are examples from a rat treated $0.1 \times LD_{50}$. A5 and B5 are examples from a rat treated $0.01 \times LD_{50}$. A large number of degenerating neurons are clearly visible in the stratum pyramidale (SP) of CA3 subfield of the rat treated with $1 \times LD_{50}$ (arrows in A2). In addition, the latter group exhibits a reduced MAP-2 immunoreactivity (B2) in comparison to the control group (B1). Scale bar = 100 μ m.

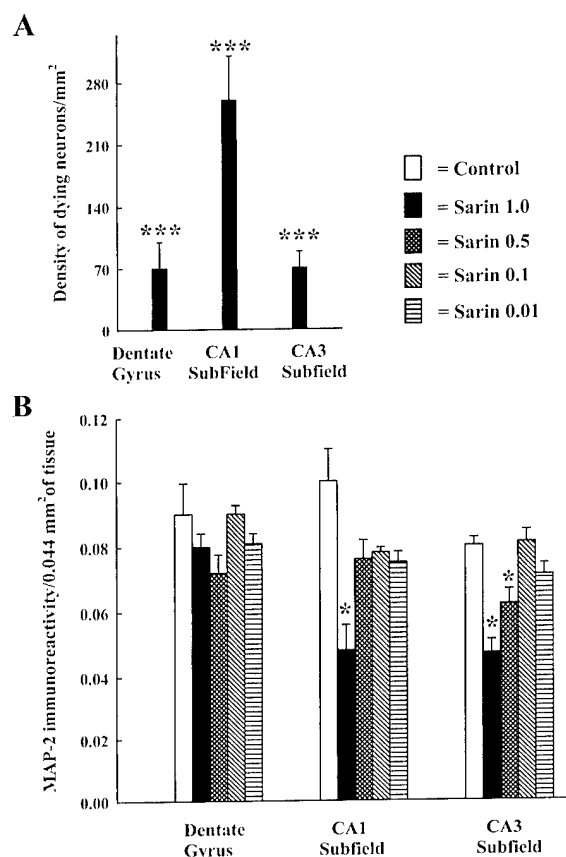


Fig. 14. Histograms in (A) show the density of dying neurons per mm² area of different cell layers in the hippocampal formation (i.e. dentate gyrus and CA1 and CA3 subfields) in control and treated groups. Histograms in (B) show MAP-2 immunoreactive elements in mm² per unit area (0.044 mm²) of the dentate gyrus and the CA1 and CA3 subfields. Animals were treated with a single intramuscular injection of 1, 0.5, 0.1 or 0.01 \times LD₅₀ of sarin in saline and analyzed at 24 h after exposure. Values represent means and standard errors ($n=5$ per group). *** $P < 0.001$; * $P < 0.05$.

tion, severe tremors, seizures, and convulsions clearly precede these changes in the brain. The above changes in the brain following 1 \times LD₅₀ sarin exposure are significant and could lead to considerable motor and sensory abnormalities, ataxia, and learning and memory deficits, as observed with exposure to other organophosphates including soman (Gardner et al., 1984; McDonald et al., 1988; Veronesi et al., 1990).

Interestingly, animals treated with 0.5, 0.1 and 0.01 \times LD₅₀ did not exhibit motor convulsions and the above-mentioned neuropathological changes except in the cerebellum, where animals treated with 0.5 \times LD₅₀ showed some Purkinje neuron loss. Thus, it appears that the initiation of acute brain damage produced by sarin is related to the sarin-induced seizures, which is consistent with the results obtained after acute soman exposure (Burchfiel and Duffy, 1982; McDonough et al., 1987; Singer et al., 1987; Nieminen et al., 1997; Kadar et al., 1992). On the other hand, the degeneration of Purkinje cells in the cerebellum with 0.5 \times LD₅₀ dose of sarin reveals that degenerative changes in the cerebel-

lum following sarin exposure can occur in the absence of seizures, suggesting that cerebellum is quite vulnerable to even lower doses of the sarin. Taken together, the above results reflect differential sensitivity of different regions of the brain to lower doses of the sarin.

Seizures and BBB disruption

In this study, the sarin-induced alteration in the BBB was evaluated using [³H]hexamethonium iodide uptake assay and immunolabeling that recognizes a BBB-related antigen, EBA (Rosenstein et al., 1992; Sternberger and Sternberger, 1987). The BBB plays a significant role in maintaining the homeostasis of the CNS microenvironment (Joo, 1996; Jensen et al., 1998). Further, the cerebrovasculature is a site of injury in a number of neurological diseases and is involved in toxicant-induced injury to the nervous system (Jacobs, 1982; Aschner and Gannon, 1994; Uno et al., 1996). A study by Nitsch and Klatzo (1983) suggested that cerebrovascular alterations can also occur following seizures. Indeed, BBB damage has been observed in many animal models of experimental seizures (McLeod et al., 1984; Bolwig, 1989; Grange-Messent et al., 1999). However, the alteration in BBB permeability is not directly dependent on AChE inhibition (Ashani and Catravas, 1981) but prolonged changes in one molecule could produce a transient change in the physiological state of the BBB (Petralli et al., 1991). Our results demonstrate dramatic increases in BBB permeability (associated with a drastic reduction in the expression of EBA in many regions of the brain including the cerebral cortex and the hippocampus at 24 h after 1 \times LD₅₀ sarin exposure. This is clearly indicative of a marked alteration in the BBB after 1 \times LD₅₀ sarin exposure. Moreover, the changes in the BBB in animals receiving 1 \times LD₅₀ were clearly associated with seizures. Alterations in the EBA staining pattern could be due to a number of alterations ranging from an overt tissue disruption and subsequent remodeling of the vasculature to more subtle changes such as degradation of the antigen or post-translational alterations influencing epitope availability (Sternberger and Sternberger, 1987; Mori et al., 1992). In contrast, animals intoxicated with the 0.5 \times LD₅₀ dose did not convulse but showed BBB damage in the brainstem and the midbrain with [³H]hexamethonium uptake assay but not with EBA analysis. This suggests that there is only a minor or transient leakage of the BBB in these regions with 0.5 \times LD₅₀ dose. The decrease in plasma BChE with 0.5 \times LD₅₀ dose in this study and the previous findings that a significant inhibition of the cholinesterase in brain capillary walls by cholinesterase inhibitors can alter the BBB permeability (Grauer et al., 2001; Skultetyova et al., 1998) also support the above interpretation. No significant changes in BBB permeability and EBA staining were observed in animals receiving 0.1 and 0.01 \times LD₅₀ sarin. Animals belonging to the latter groups also did not exhibit seizures. Thus, a significant and prolonged disruption of the BBB (evidenced by contemporaneous increases in BBB permeability and decreases in EBA immunoreactive elements) after sarin exposure is clearly dose-dependent and

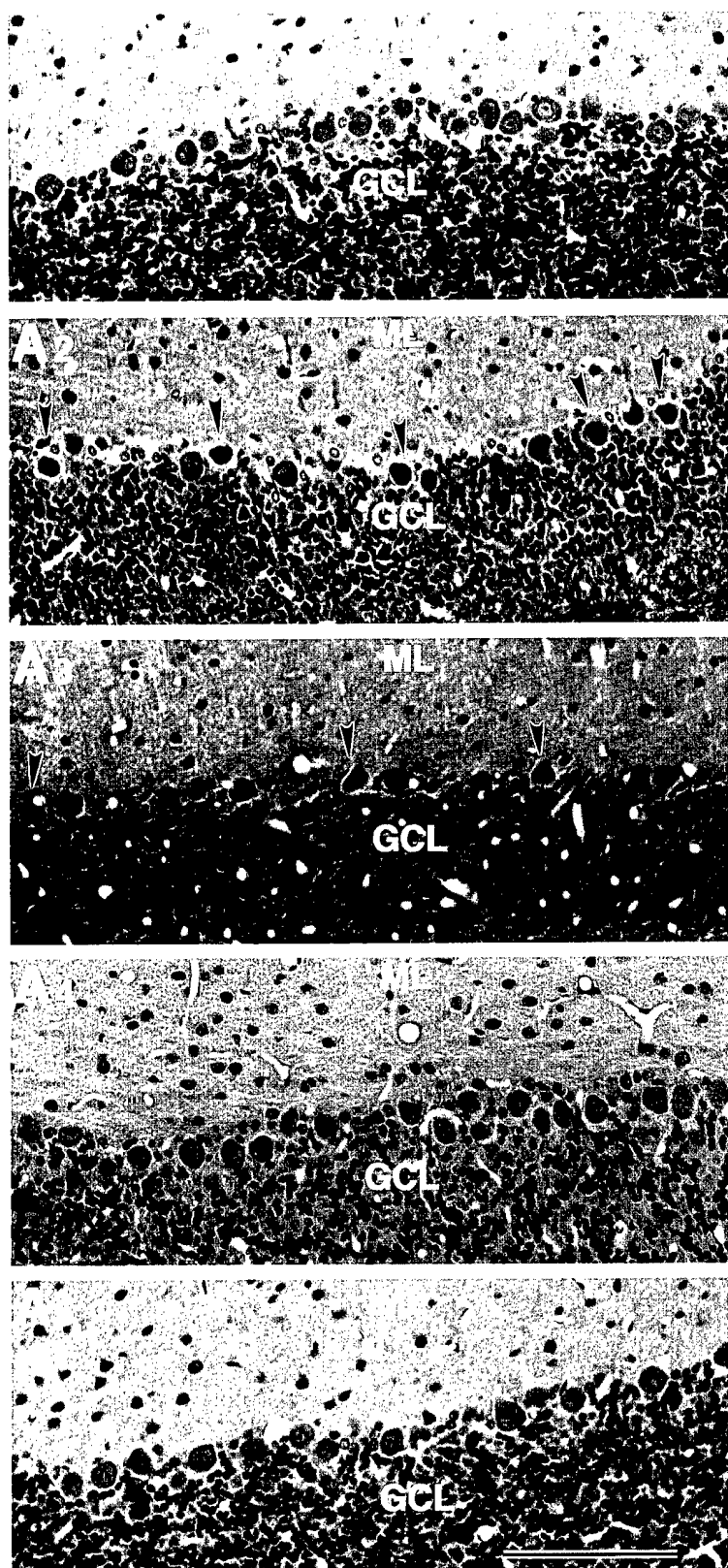


Fig. 15. Alterations in the cerebellum at 24 h following sarin exposure. Values represent means and standard errors ($n = 5$ per group). A1–A5. H&E staining of lobule 2 of the cerebellar vermis. A1 shows an example from a control rat. A2 illustrates an example from a rat treated with $1 \times LD_{50}$ sarin. A3 shows an example from a rat treated with $0.5 \times LD_{50}$ sarin. A4 and A5 show examples from rats treated with 0.1 and $0.01 \times LD_{50}$ sarin, respectively. A large number of degenerating Purkinje neurons are clearly visible in the Purkinje cell layer of rats treated with either 1 or $0.5 \times LD_{50}$ (arrows in A2 and A3). Scale bar = $100 \mu\text{m}$.

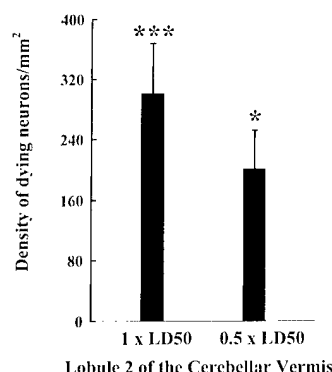


Fig. 16. Histograms show the density of dying neurons per mm² area of the Purkinje cell layer in lobule 2 of the cerebellar vermis. Animals were treated with a single intramuscular injection of 1, 0.5, 0.1 and 0.01 \times LD₅₀ of sarin and analyzed at 24 h after the exposure. Values represent means and standard errors ($n=5$ per group). *** $P < 0.001$; * $P < 0.05$.

appears to be coupled to the development of seizures after the sarin exposure.

Pattern of brain injury induced by sarin

The results of this study indicate that an acute sarin exposure at $1 \times \text{LD}_{50}$ can result in extensive damage to the adult brain, particularly involving the motor and somatosensory cortex, the hippocampus, and the cerebellum. The rats displaying the latter neuropathology also had seizures and BBB damage. The pattern of neuropathological change depends upon a number of factors such as dosage, duration of the exposure, age of the animal, and species (Storm-Mathisen, 1970; McLeod et al., 1984; McDonough et al., 1989; Veronesi et al., 1990). The neuropathological changes observed in surviving rats treated with $1 \times \text{LD}_{50}$ sarin in this study is somewhat similar to those observed in rats following lethal doses of soman, a military nerve agent, and following subchronic exposure to anticholinesterases (Storm-Mathisen, 1970; McLeod et al., 1984; McDonough et al., 1989; Veronesi et al., 1990). The neuropathology in distinct brain regions as described above is likely the result of ischemia or a direct neurotoxic action attributed to the seizures associated with nerve agents (Millis et al., 1988; Carpentier et al., 1990). Thus, the pattern of brain injury induced by $1 \times \text{LD}_{50}$ sarin has similarities to those described after $1 \times \text{LD}_{50}$ soman and other anticholinesterases.

Extent of alterations in MAP-2 expression after sarin exposure

An important neuronal component, MAP-2, is enriched in dendrites and cell bodies of neurons (Tucker et al., 1988; Gavin, 1997). The MAP-2 plays a key role as a structural protein necessary for the maintenance of cytoskeleton integrity and in neuronal growth, plasticity, and regeneration (Johnson and Jope, 1992), and also aids in dendritic remodeling and post-synaptic changes that occur after certain lesions. The present results suggest that acute $1 \times \text{LD}_{50}$ sarin exposure induces a significant

decrease in the expression MAP-2 in dendrites of neurons belonging to the cerebral cortex and CA1 subfield of the hippocampus. These changes were clearly evident by 24 h after the injury. However, with lower doses of sarin, such changes were not observed. Further, in control animals and animals treated with lower doses of sarin, MAP-2-positive dendrites in the cerebral cortex and CA1 subfield of the hippocampus appeared as long, branching processes. After $1 \times \text{LD}_{50}$ sarin injury, MAP-2-positive dendrites not only reduced in number but also exhibited a fragmented appearance with swelling occurring along the length of individual dendrites. Several studies suggest that degradation of MAP-2 following exposure to neurotoxic chemicals in the cerebral cortex and the hippocampus is a result of global ischemia (Inuzuka et al., 1990; Matesic and Lin, 1994), or excitotoxic cascade of biochemicals, which could initiate a massive cell depolarization involving glutamate release and activation of *N*-methyl-D-aspartate receptors (Fineman et al., 1993). Activation of calcium calmodulin kinase II is another possibility, which may result in abnormal phosphorylation of phosphoproteins such as MAP-2 and thereby impair their normal structure and function of neurons (Folkerts et al., 1998). Thus, in the present study, a significantly decreased MAP-2 staining following $1 \times \text{LD}_{50}$ sarin could be due to abnormal phosphorylation of phosphoproteins in the surviving neurons. Abnormal phosphorylation of MAP-2 can occur after depolymerization of microtubules within dendrites and lead to cytoskeletal alterations (Folkerts et al., 1998). Further, with time, the early cytoskeletal alterations observed after $1 \times \text{LD}_{50}$ sarin exposure can lead to disruption of neuron and synaptic structure, axonal transport, and ultimately motor and cognitive processes.

Relationship between neuronal injury and neurochemical changes

The initiation of sarin-induced seizures and convulsions appeared to be due to cholinergic and non-cholinergic effects. The cholinergic system in the CNS plays an important role in learning and memory and has a regulatory role in certain neurobehavioral functions (Lyeth et al., 1990; Levey et al., 1991; Nieminen et al., 1997). Previous studies have reported that sarin may exert its cholinergic effects directly by the activation of limbic AChRs and accumulation of endogenous ACh following ChE inhibition in the PNS or CNS, which is sufficient to cause severe seizures followed by neurodegenerative processes (Taylor, 1985, 1996). Our data suggest that acute treatment with sarin at 1.0 and $0.5 \times \text{LD}_{50}$ causes a significant inhibition in the plasma BChE as well as the brain AChE. This inhibition may be responsible for the differential regulation of the receptors activated directly by ACh.

We also measured m2 muscarinic receptors in this study because effects on mAChRs can play a role in the toxicity of certain organophosphate compounds, as these receptors are coupled to different G-proteins to induce cellular signaling from the cell surface. The m2 mAChR is coupled to Gi protein, and hence activation

of m2 mAChRs can lead to inhibition of the adenylate cyclase (Abdallah et al., 1992; Huff et al., 1994). It has been also suggested that the toxicity of organophosphate anticholinesterases have actions on both nAChRs and mAChRs when their concentrations in circulation rise above micromolar levels. However, at nanomolar concentrations many organophosphates as well as the nerve gas agents such as soman and sarin can selectively affect a small population of mAChRs (Bakry et al., 1988; Silveira et al., 1990; Katz et al., 1997). Thus, a differential regulation of m2 muscarinic receptors in different regions may lead to a differential response leading to inhibition of the adenylate cyclase (Headley and Grillner, 1990). In this context, the sarin-induced reductions in mACh receptors in the cerebrum, and increases in mACh receptors in the midbrain, brain stem and the cerebellum, could transduce a differential response in these brain regions. The decrease of mAChRs in the cortex is consistent with the receptor plasticity observed following exposure to organophosphate compounds (Jett et al., 1994). However, the increase in other brain regions in this study is intriguing. The increase in binding parameters is typically related to the absence of the natural ligand or to the effect of chronic treatment with an antagonist. However, in this study, the level of natural ligand has been increased with sarin exposure and also there was no treatment with antagonists. In this context, it appears that the differential effect of sarin exposure on mACh receptors in different regions of the brain reflects region-specific response of the mACh receptors to the inhibition of AChE.

Sarin may also exert its effects through other cholinergic mechanisms, which can interact directly with mACh receptors (Rocha et al., 1998; Chebabo et al., 1999), or pre-synaptic muscarinic receptors by reducing action potential-dependent release of GABA in the post-synaptic neuron (Chebabo et al., 1999). Alternatively, sarin may exert its effect through non-cholinergic mechanisms, which can lead to neuronal cytotoxicity similar to what is observed after the soman exposure (John and Brian, 1993; Somani, 1992; Fernando et al., 1984). Taken together, alterations in the expression of mACh receptors observed in this study after $1 \times LD_{50}$ sarin exposure could lead to a number of physiological shifts

in the brain which in turn can lead to behavioral abnormalities.

CONCLUSIONS

This novel study evaluated the early changes in the adult brain after a single exposure to different doses of the sarin. The results demonstrate that a single exposure to sarin at $1 \times LD_{50}$ causes seizures and leads to an extensive brain damage by 24 h involving the cerebral cortex, the hippocampus (dentate gyrus, and CA1 and CA3 subfields) and the cerebellum. This was characterized by a significant inhibition of brain regional AChE and m2 mAChR ligand binding in the cerebrum, a dramatic increase in the BBB permeability with a severe decrease in EBA expression, a diffuse neuronal cell death coupled with a decrease in MAP-2 expression within dendrites of surviving neurons. The above changes in the brain following $1 \times LD_{50}$ sarin exposure are considerable and could lead to a substantial motor and sensory abnormalities, ataxia, and learning and memory deficits. However, animals treated with 0.5, 0.1 and $0.01 \times LD_{50}$ exhibited neither seizures nor the above-mentioned combination of neuropathological changes. This may suggest that the early brain damage after acute exposure to sarin is clearly dose-dependent and brain pathology is clearly apparent with only $1 \times LD_{50}$ exposure. However, this study does not rule out long-term changes after lower doses of the sarin exposure, as pathological changes in the brain are usually not pronounced with milder injury during the early post-injury period. Thus, analyses of the long-term effects of different doses of the sarin on morphological, neurochemical, and behavioral alterations in the brain remain interesting and important issues for future studies.

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Sensorimotor Deficit and Cholinergic Changes following Coexposure with Pyridostigmine Bromide and Sarin in Rats

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A myriad of neurological symptoms including muscle and joint pain, ataxia, chronic fatigue, headache, and difficulty in concentration have been reported by Persian Gulf War (PGW) veterans. A large number of these veterans were prophylactically treated with pyridostigmine bromide (PB) and possibly exposed to sarin. In the present study we investigated the effects of PB and sarin, alone and in combination, on sensorimotor performance and the central cholinergic system of rats. Male Sprague-Dawley rats were treated with PB (1.3 mg/kg, 15 daily doses, oral) and sarin (50, 75, 90, and 100 μ g/kg, single im dose on day 15), alone and in combination. The animals were evaluated for postural reflexes, limb placing, orienting to vibrissae touch, incline plane performance, beam-walk time, and forepaw grip time 7 and 15 days following treatment with sarin. Treatment with either PB or sarin alone resulted in significant sensorimotor impairments. Coexposure to sarin and PB resulted in significant sensorimotor deficits that worsened over time. By 15 days following sarin treatment, plasma butyrylcholinesterase (BChE) activity returned to normal levels in the animals treated with sarin alone, whereas in the animals exposed to PB or PB plus sarin, there was an increase in the enzyme activity. Cortical acetylcholinesterase (AChE) activity remained inhibited in the animals treated with sarin alone and in combination with PB. Muscarinic acetylcholine receptor (m2 mAChR) ligand binding with [³H]AFDX-384 in cortex and brain stem showed significant increases (~120–130% of control) following coexposure to PB and sarin at higher doses. To evaluate the potential of PB for augmentation or inhibition of the toxicity induced by acute sarin exposure, the animals were exposed to either 10 or 100 μ g/kg sarin (single im injection) with or without pretreatment with PB, and sacrificed 3 h after treatment with sarin. Pretreatment with PB offered slight protection in the plasma as well as brain regional enzyme activities. Pretreatment with PB did not have any effect on sarin-inhibited brain regional AChE activity following treatment with 100 μ g/kg sarin. These results show that prophylactic treatment with PB offers some degree of protection in peripheral cholinesterase. Furthermore, these results show that treatment with either sarin or PB alone resulted in sensorimotor impairments, while coexposure to high doses of sarin with PB caused an exacerbated deficit.

Key Words: sarin; pyridostigmine bromide; PB; acetylcholinesterase; muscarinic acetylcholine receptor; neurotoxicity; combined exposure; sensorimotor; Gulf War.

Since their return from the war, many Persian Gulf War (PGW) veterans have complained of symptoms including chronic fatigue, muscle and joint pain, ataxia, rash, headache, difficulty concentrating, forgetfulness, and irritability (Institute of Medicine, 1995, 2000), as well as sensorimotor complaints including numbness or tingling, weakness, and heaviness of the arms and legs (Knoke *et al.*, 2000). These veterans were exposed to a unique combination of biological, chemical, and psychological environments (Caldwell, 1992; Institute of Medicine, 1995, 2000). Combinations of chemical exposures included a variety of pesticides and nerve agent sarin (Institute of Medicine, 1995, 2000; McCauley *et al.*, 2001). In addition, a majority of U.S. service personnel were given pyridostigmine bromide (PB) as a prophylactic against possible nerve gas attack (Cook *et al.*, 1992; Golomb, 1999). Sarin, an organophosphate agent, and PB, a quaternary dimethyl carbamate, primarily affect the cholinergic system. PB binds to peripheral cholinesterase and thus shields the enzyme from sarin-induced inhibition.

PB has been used as a treatment for myasthenia gravis at a higher dose range than what was given to Persian Gulf War Veterans (Breyer-Pfaff *et al.*, 1985, 1990). The veterans were given a course of twenty-one 30-mg tablets of PB as prophylaxis against organophosphate (OP) nerve agents (Institute of Medicine, 1995, 2000; Persian Gulf Veterans Coordinating Board, 1995). At this dose, PB reversibly inhibits 30–40% of the AChE in the peripheral nervous system, thus limiting irreversible inhibition of the enzyme by nerve agents (Blick *et al.*, 1991). AChE activity is restored following spontaneous decarbamylation resulting in near normal neuromuscular and autonomic functions (Blick *et al.*, 1991; Watts and Wilkinson, 1977). Toxic symptoms associated with PB overdose result from overstimulation of nicotinic and muscarinic receptors in the peripheral nervous system, causing exaggerated cholinergic

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effects such as muscle fasciculations, cramps, weakness, muscle twitching, tremor, respiratory difficulty, gastrointestinal tract disturbances, and paralysis (Abou-Donia *et al.*, 1996; Cook *et al.*, 1992). With severe intoxication, death may occur because of asphyxia. The positive charge on the quaternary pyridinyl nitrogen prevents PB from crossing the intact blood-brain barrier (BBB) (Birtley *et al.*, 1966). Therefore, central nervous system (CNS) effects of PB are not expected unless BBB permeability is compromised. Approximately half of U.S. personnel seen at the health care clinics during the PGW complained of muscarinic symptoms that may have been related to chemical exposure, including PB (Cook *et al.*, 1992; Golomb, 1999). The role of PB in the development of signs and symptoms associated with PGW deployment remains poorly understood.

Sarin, O-isopropylmethylphosphonofluoridate, is an organophosphate that has been studied primarily as a potent warfare nerve agent (Taylor, 1985). The main clinical features associated with acute sarin intoxication are seizures, fasciculations, tremors, and hypothermia (Taylor, 1985). The appearance of these symptoms correlates with the inhibition of AChE, both in the CNS and peripheral nervous system (PNS) (Gupta *et al.*, 1991). This is followed by excessive accumulation of acetylcholine, leading to hyperactivation of nicotinic and muscarinic acetylcholine receptors. Excessive accumulation of acetylcholine leads to activation of ligand-gated ion channel, nicotinic acetylcholine receptor (nAChR), and muscarinic acetylcholine receptor (mAChR). These receptors mediate diverse cellular responses by distinct signaling mechanisms (Wess, 1996). Indeed, previous studies from our laboratory and others have shown that organophosphate compounds, including sarin, cause differential regulation of nAChR and mAChR (Huff *et al.*, 1994; Jett *et al.*, 1991; Katz *et al.*, 1997; Khan *et al.*, 2000). *In vitro* studies by Bakry *et al.* (1988) suggest that sarin binds to nAChR and modulates its ligand-binding characteristics. Therefore, it is likely that changes in cholinergic pathways play a key role in the toxicity induced by sarin.

In addition to acute cholinergic effects, healthy individuals exposed to low-dose sarin have been reported to exhibit neurological signs and symptoms up to 10 years following initial exposure (Duffy and Burchfiel, 1980; Sidell, 1974). The neurological effects included increased beta activity and increased amount of rapid eye movement sleep (Duffy and Burchfiel, 1980). Abnormal electrophysiological recordings following a single large dose or repeated subclinical doses of sarin in rhesus monkeys have been observed (Burchfiel *et al.*, 1976; Burchfiel and Duffy, 1982). Distal sensory axonopathy has been observed 3 years after sarin intoxication in Tokyo (Himuro *et al.*, 1998). Repeated inhalation exposure to sarin has been shown to cause muscular weakness of the limbs in mice (Husain *et al.*, 1993). Long-term behavioral changes characterized by a decrease in activity, increased morbidity, and changes in gait have been observed in rats following low-level sarin exposure (Kassa *et al.*, 2001).

In the present study, we evaluated the sensorimotor performance and brain regional acetylcholinesterase activities and m2 mAChR ligand binding following treatment with sarin and PB, alone and in combination. These results suggest that treatment with sarin and PB alone resulted in sensorimotor impairment and cholinergic changes and that coexposure of high doses of sarin with PB resulted in exacerbated deficits.

MATERIALS AND METHODS

Sarin stock (1.90 mg/ml in saline) was obtained from the U.S. Army Medical Research and Materiel Command, Fort Detrick, Maryland. Pyridostigmine bromide, butyrylthiocholine iodide, and acetylthiocholine iodide were obtained from Sigma Chemical Co. (St. Louis, MO). [3 H]AF-DX384[2,3 Dipropylamino] (sp. activity, 100 Ci/m mol) was obtained from NEN (Boston, MA). All other reagents were of highest purity available commercially.

Male Sprague-Dawley rats (200–250 g) were obtained from Zivic-Miller Laboratories (Allison Park, PA) and housed in the Duke University Medical Center vivarium on 12-h dark-light cycle. The animals were allowed food and water *ad libitum*. The animals were treated with PB or water or sarin between 7:30 and 11:00 AM. All animal treatments and procedures were carried out strictly according to the recommended guidelines by the Army and the Duke University Medical Center institutional animal care and use committee.

To evaluate the neurobehavioral and biochemical effects of exposure to sarin and PB, two different sets of treatments were carried out.

Treatment 1

Animals in this treatment were subjected to neurobehavioral evaluations on days 7 and 15 following treatment with sarin and finally sacrificed on day 15 for biochemical determinations.

Control: Animals ($n = 5$) received daily water (orally by gavage) for 15 days and then a single im injection of normal saline on day 15. PB alone: animals in this group ($n = 5$) received PB (1.3 mg/kg orally by gavage) for 15 days and a single im injection of normal saline on day 15.

Sarin alone: animals in this group ($n = 10$) received water (orally by gavage daily for 15 days), and on day 15 they were treated with single im dose of 50, 75, 90, and 100 μ g/kg, sarin (0.50, 0.75, 0.90, and $1 \times \text{LD}_{50}$, respectively). There were 15 animals in the group treated with 100 μ g/kg sarin.

PB and sarin: the animals received pretreatment with PB (1.3 mg/kg, daily orally by gavage) for 15 days and then single im treatment with 50, 75, 90, and 100 μ g/kg sarin on day 15. The number of animals treated in each group was the same as for sarin alone.

Treatment 2

Animals were sacrificed 3 h following treatment with sarin for biochemical determinations only.

Control: the animals ($n = 5$) received water daily (orally by gavage) for 15 days and then a single im injection of normal saline on day 15.

PB alone: animals ($n = 5$) received PB (1.3 mg/kg, oral) for 15 days and a single im injection of normal saline on day 15.

Sarin alone: animals in this group ($n = 5$) received water (orally by gavage daily for 15 days) and on day 15 they were treated with single im dose of 10 and 100 μ g/kg sarin (0.10 and $1 \times \text{LD}_{50}$, respectively). There were 10 animals in the group treated with 100 μ g/kg sarin.

PB and sarin: the animals received pretreatment with PB (1.3 mg/kg daily orally by gavage) for 15 days and then single im treatment with 10 and 100 μ g/kg sarin on day 15. The number of animals treated in each group was the same as for sarin alone.

At the termination of the experiment, the animals in both the treatment schedules were anesthetized with 0.2 ml ketamine/xylazine (100 mg/kg ket-

amine, 15 mg/kg xylazine), and blood was drawn in a heparinized syringe. Brains were removed and washed thoroughly with ice-cold normal saline to remove blood. Brain regions, cortex, midbrain, cerebellum, and brain stem were dissected on ice and snap frozen in liquid nitrogen. Plasma was separated and frozen at -80°C for enzyme studies.

Behavioral Testing Battery

The behavioral tests employed in these studies evaluate sensorimotor reflexes, motor strength, and coordination. All behavioral testing was performed by an observer blind to the animal's treatment status and was carried out in a soundproof room with subdued lighting (less than 10.76 lumens/m², ambient light) between 7 and 11:30 AM. Behavioral testing was carried out 7 and 15 days after sarin was administered.

Reflexes. Postural reflexes (Bederson *et al.*, 1986; Markgraf *et al.*, 1992), visual, tactile, and proprioceptive forelimb-placing responses (Markgraf *et al.*, 1992), and orienting to vibrissae touch (Whishaw *et al.*, 1985) were carried out as described by Abou-Donia *et al.* (2001).

Inclined plane. Rats were placed on a flat plane in the horizontal position, with the head facing the side of the board to be raised (Abou-Donia *et al.*, 2001; Yonemori *et al.*, 1998). The angle at which the rat began to slip downward was recorded. The results of the two trials were averaged for each testing session.

Forepaw grip time. The rats' forepaw strength was assessed by having them grip a 5-mm diameter wood dowel that was held horizontally and raised so that the rat supported its body weight, as described by Andersen *et al.* (1991) and Abou-Donia *et al.* (2001). Time to release grip was recorded in seconds. The results of the two trials were averaged for each testing session.

Beam-walking. The testing apparatus was a 2.5×122 cm wooden beam elevated 75.5 cm above the floor, with wooden supports as described by Goldstein (1993) and Abou-Donia *et al.* (2001). Beam-walking ability was measured with a seven-point scoring system scale as previously described by Goldstein (1993, 1995): 1, the rat is unable to place the affected hindpaw on the horizontal surface of the beam; 2, the rat places the affected hindpaw on the horizontal surface of the beam and maintains balance for at least 5 s; 3, the rat traverses the beam while dragging the affected hindpaw; 4, the rat traverses the beam and at least once places the affected hindpaw on the horizontal surface of the beam; 5, the rat crosses the beam and places the affected hindlimb on the horizontal surface of the beam to aid less than half its steps; 6, the rat uses the affected hindpaw to aid more than half its steps; and 7, the rat traverses the beam with no more than two foot slips. In addition, the latency until the animal's nose enters the goal box (up to 90 s) is recorded for the final trial. Rats that fell off the beam or did not enter the goal box were assigned latencies of 90 s.

Statistical analyses. Comparisons across treatment groups for postural reflexes, limb placing, and vibrissae touch orientation were analyzed with nonparametric analysis of variance (Kruskal-Wallis test). Data for the remaining behavioral tests were compared among groups by one-way or two-way repeated measures ANOVA as appropriate. A three-way repeated measures ANOVA was used to compare the effects of increasing doses of sarin alone or in combination with a fixed dose of PB. If a significant difference was found, Fisher's LSD tests were applied to permit post hoc pairwise comparisons. A two-tailed p value of < 0.05 was considered statistically significant.

Enzyme and Receptor Assay

Cholinesterase determination. AChE in brain regions and BChE in plasma activities were determined according to the method of Ellman *et al.* (1961) modified for assay in a Molecular Devices UV Max Kinetic Microplate Reader as previously described (Abou-Donia *et al.*, 1996; Khan *et al.*, 2000). Protein concentration was determined by BCA method according to Smith *et al.* (1985). The enzyme activities are expressed as micromoles substrate hydrolyzed per minute per milligram protein for brain regions and nanomoles

substrate hydrolyzed per minute per milligram protein for plasma (percent of control).

Muscarinic acetylcholine receptor (mAChR) binding assay. For the assay of mAChR, the tissue was homogenized in 10 mM phosphate buffer, pH 7.4, and centrifuged at $40,000 \times g$ for 10 min; the membranes were suspended in the same buffer at the protein concentration of 1.5–2.5 mg/ml as described by Huff *et al.* (1994). The m2 mAChR binding was carried out by using m2-selective ligand [³H] AFDX 384 as described earlier (Slotkin *et al.*, 1999; Khan *et al.*, 2000). The results are expressed as specific binding (dpm)/mg protein (percent of control).

Statistical analysis. The results were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test; p value < 0.05 was considered significant.

RESULTS

Clinical Signs

The animals were observed for the development of clinical signs of toxicity. Treatment with 100 $\mu\text{g/kg}$ sarin resulted in convulsions and cholinergic toxicity. The onset and magnitude of seizure was greater in the animals treated with a combination of PB and sarin (100 $\mu\text{g/kg}$). There was no mortality in the group of animals treated with 50 or 75 $\mu\text{g/kg}$ sarin, alone or in combination with PB. Treatment with 100 $\mu\text{g/kg}$ sarin alone resulted in 7 deaths out of 15 animals, whereas in the group pretreated with PB, only 5 animals died out of 15. Similarly, 2 animals out of 10 died in 90 $\mu\text{g/kg}$ sarin alone treatment group as compared with only 1 in the group treated with 90 $\mu\text{g/kg}$ sarin and PB.

Behavioral Results

Figures 1 and 2 give the effects of PB and dose-response comparisons for rats treated with increasing doses of sarin and increasing doses of sarin with a fixed dose of PB for each behavioral test on days 7 and 15, respectively, following treatment with sarin. Control rats began to slip off the incline plane when it was raised to 60° from horizontal. Forepaw grip time improved by an average of 3 s between the two testing sessions in controls. Beam-walk scores were perfect for all control rats at both time points. Beam-walk times decreased by an average of 4 s between the two testing days in controls.

Each treatment (PB, sarin, and PB + sarin) resulted in significant sensorimotor impairments compared with controls, as reflected in each behavioral test at each time point. Treatment with PB alone resulted in a greater behavioral impairment than sarin alone only for grip time and only on day 7 (Fig. 1, lower panel). Exposure to the combination of PB and sarin did not result in further deterioration in grip time than that caused by treatment from PB alone. However, the combination of PB and sarin resulted in greater impairment than sarin alone for incline plane performance on both days 7 and 15 and for forepaw grip on day 7 (Figs. 1 and 2).

For rats treated with sarin alone, there was a dose \times time interaction for incline plane performance and grip time, indi-

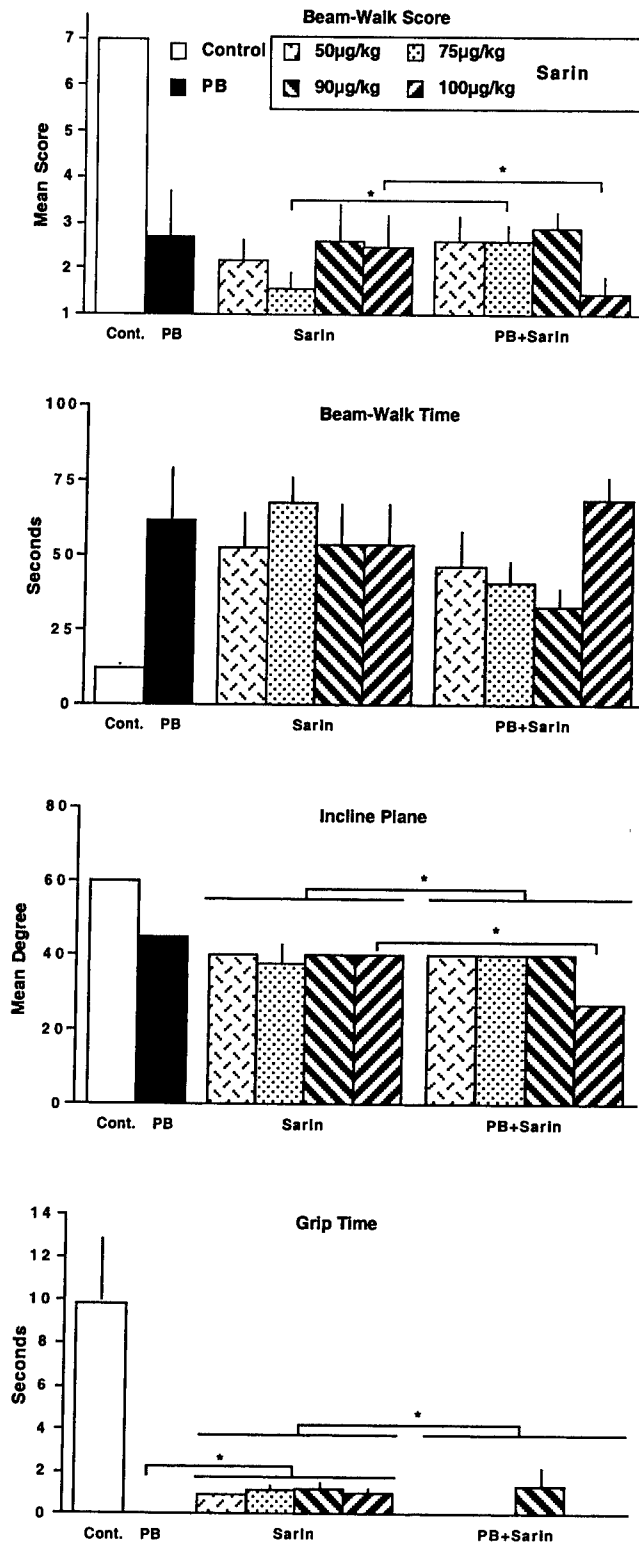


FIG. 1. Effect of treatment with sarin and PB, alone or in combination, on sensorimotor performance on day 7 following treatment with single im injection of various doses of sarin. The animals were pretreated with PB (1.3 mg/kg, oral, daily for 15 days) and various doses of single im injection of sarin (50, 75, 90, and 100 µg/kg). The animals were tested for beam-walk score, beam-walk time, incline plane, and grip response. Each treatment (PB, sarin, and PB + sarin) resulted in significant impairments compared with controls on each behavioral test at each dose of sarin. The data are presented as mean \pm SE, $n = 5$. *Indicates statistically significant.

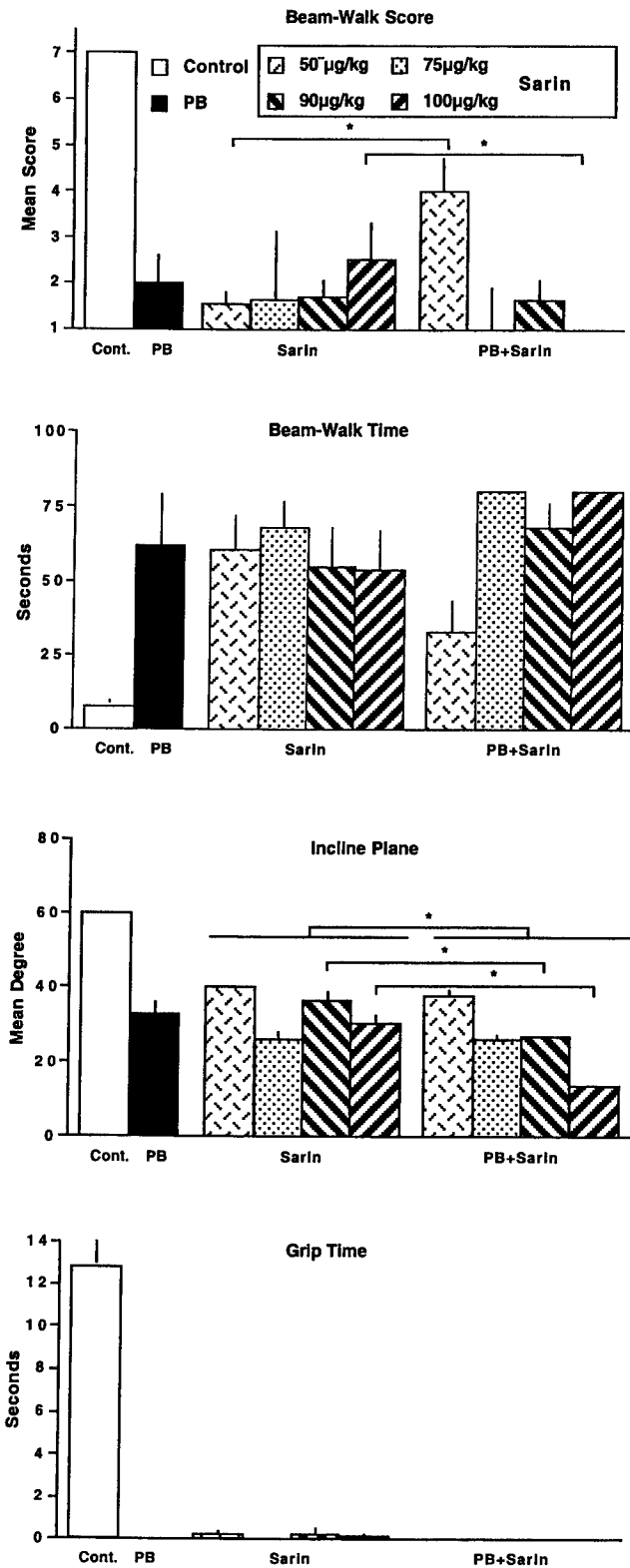


FIG. 2. Effect of treatment with sarin alone on sensorimotor performance on day 15 following treatment with single im injection of various doses of sarin. The animals were treated with various doses of single im injection of sarin (50, 75, 90, and 100 µg/kg). The animals were examined blindfolded for beam-walk score, beam-walk time, incline plane, and grip response. Each treatment (PB, sarin, and PB + sarin) resulted in significant impairments compared with controls on each behavioral test at each dose of sarin. The data are presented as mean \pm SE, $n = 5$. *Indicates statistically significant.

cating poorer performance on these tasks on day 15 versus day 7 for at least one dose. For incline plane, rats that had received 75 or 100 μg sarin had significantly poorer performance on day 15 compared with day 7. For grip time, the difference between the two time points was significant only for rats that had received 75 μg sarin. For the rats treated with PB + sarin, there were significant dose \times time interactions for each behavioral task (Figs. 1 and 2).

Three-way repeated measures ANOVA comparing increasing doses of sarin with increasing doses of PB + sarin showed significant differences for both incline plane performance and beam-walking scores. For incline plane performance, rats given PB + 90 μg sarin had poorer performance than those given 90 μg sarin alone, but only on day 15 (Fig. 2). The combination (PB + 100 μg sarin) resulted in poorer incline plane performance versus the 100- μg dose of sarin given alone on both days 7 and 15 (Figs. 1 and 2). For beam-walking score, the combination of PB + sarin resulted in improved performance at the 50- μg dose of sarin on day 15 only (Fig. 2) and at the 75- μg dose on day 7 only (Fig. 1). In contrast, the 100- μg dose of sarin resulted in poor beam-walk scores at both time points (Figs. 1 and 2).

Effect of Treatment with Sarin and PB, Alone and in Combination, on Plasma Cholinesterase Activity

Sarin exposure results in inhibition of plasma cholinesterase at various levels depending upon the dose and duration of exposure. The data presented in Figure 3A are from the animals 15 days after treatment with single im injection of 50, 75, 90, and 100 $\mu\text{g}/\text{kg}$ sarin alone and in combination with 15 days of pretreatment with oral PB. Treatment with PB alone resulted in a significant elevation in the enzyme activity ($\sim 160\%$ of control). A combination of treatment with a 100- μg dose of sarin and PB also resulted in a significant increase ($\sim 126\%$ of control) in the plasma enzyme activity. To study the effect of PB on acute treatment with sarin, in a separate set of experiments we evaluated plasma cholinesterase activity 3 h following treatment with either 10 μg or 100 $\mu\text{g}/\text{kg}$ single im injection of sarin alone or in combination with pretreatment with 15 days of daily oral doses of PB (Fig. 3B). The experiments at 3 h after exposure with sarin were carried out to evaluate any protection by PB of sarin toxicity involving the cholinergic system during acute exposure. Animals treated with 100 $\mu\text{g}/\text{kg}$ sarin exhibited a significant decrease in plasma cholinesterase activity ($\sim 37\%$ of control) that was less following coexposure with PB ($\sim 67\%$ of control). There was no significant change in the enzyme activity in the animals treated with 10 $\mu\text{g}/\text{kg}$ sarin, either alone or in combination with PB. These results suggest that pretreatment with PB provided some protection in plasma cholinesterase activity in rats treated with higher-dose sarin following acute exposure.

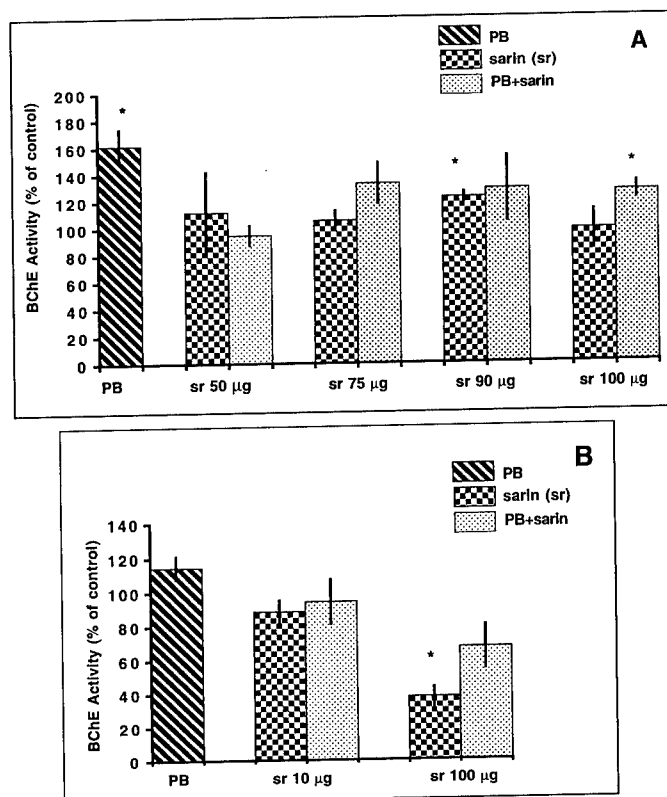


FIG. 3. Effect of treatment with sarin and PB, alone or in combination, on plasma BChE activity. Top panel (A): The animals were pretreated with PB (1.3 mg/kg, oral, daily for 15 days) and various doses of single im injection of sarin (50, 75, 90, and 100 $\mu\text{g}/\text{kg}$) and sacrificed on day 15 following treatment with sarin. Bottom panel (B): The animals were pretreated with PB (1.3 mg/kg, oral, daily for 15 days) and single im injection of sarin (10, and 100 $\mu\text{g}/\text{kg}$) and sacrificed 3 h following treatment with sarin. The control activity was 3 ± 0.1 nmoles butyrylthiocholine hydrolyzed/min/mg protein. Data are presented as mean \pm SE (% control), $n = 5$. *Indicates statistically significant.

Effect of Treatment with Sarin and PB, Alone and in Combination, on Brain Regional AChE

The data presented in Figures 4–7 show the inhibition pattern of AChE in cortex, brain stem, midbrain, and cerebellum after single im injection of 50, 75, 90, and 100 $\mu\text{g}/\text{kg}$ sarin. Data in panels 4A–7A are from the animals 15 days after treatment with sarin with and without pretreatment with 15 days of daily oral doses of PB. The data in panels 4B–7B are from the animals 3 h after treatment with 10 or 100 $\mu\text{g}/\text{kg}$ single-dose sarin, with and without pretreatment with 15 days of daily oral doses with PB. The enzyme activity remained significantly inhibited in the cortex (~ 43 – 69% of control) 15 days after treatment with 50, 75, 90, and 100 $\mu\text{g}/\text{kg}$ sarin (Fig. 4A). Pretreatment with 15 daily oral doses of PB did not have any effect on sarin-inhibited cortical AChE activity, as the enzyme activity in the animals treated with sarin and PB remained significantly inhibited compared with the control. PB treatment alone caused a significant increase in the cortex AChE ($\sim 162\%$ of control). These data further underscore the

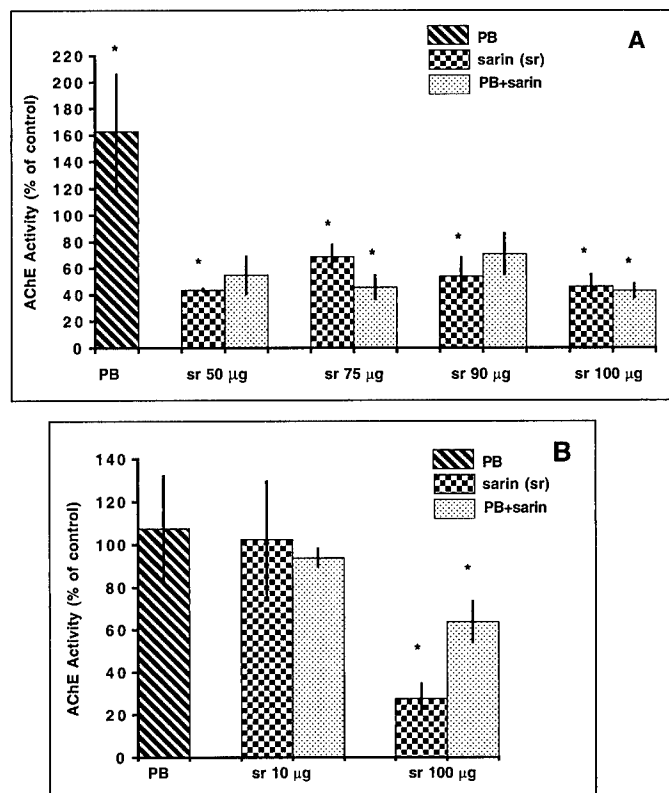


FIG. 4. Effect of treatment with sarin and PB, alone or in combination, on cortex AChE activity. Top panel (A): The animals were pretreated with PB (1.3 mg/kg, oral, daily for 15 days) and various doses of single im injection of sarin (50, 75, 90, and 100 µg/kg) and sacrificed on day 15 following treatment with sarin. Bottom panel (B): The animals were pretreated with PB (1.3 mg/kg, oral, daily for 15 days) and single im injection of sarin (10 or 100 µg/kg) and sacrificed 3 h following treatment with sarin. The control activity was 40.1 ± 9.05 nmoles acetylthiocholine hydrolyzed/min/mg protein. Data are presented as mean \pm SE (% control), $n = 5$. *Indicates statistically significant.

potential of sarin to inhibit any increase in the enzyme activity in response to treatment with PB. Data in Figure 4B emphasize that acute sarin treatment with 100 µg/kg sarin caused a significant inhibition in the enzyme activity and that PB pretreatment resulted in significantly less inhibition in the cortical enzyme activity than treatment with sarin alone. However, the enzyme activity in the animals coexposed with sarin and PB still remained significantly inhibited. Brain stem AChE activity following treatment with 50, 75, 90, and 100 µg/kg sarin, alone or in combination with 15 days of oral pretreatment with PB, are shown in Figure 5A. The enzyme activity showed a significant increase at all the doses treated with sarin alone or in combination with PB (~120–145% of control). The data in Figure 5B indicate that acute treatment with 100 µg/kg sarin did cause a significant inhibition in brain stem AChE activity and that PB pretreatment resulted in significantly less inhibition in the enzyme activity in the animals coexposed to 100 µg/kg sarin and PB than those treated with sarin alone. However, the enzyme activity in the animals coexposed to sarin and

PB still remained significantly inhibited (~63% of control). A similar pattern of significantly increased AChE activity following treatment with 50, 75, 90, and 100 µg/kg sarin, alone or in combination with 15 days of oral pretreatment with PB, was observed in midbrain (Fig. 6A) and cerebellum (Fig. 7A), suggesting a differential effect of sarin or combination of sarin with PB on brain stem, midbrain, and cerebellum compared with cortex following 15 days after treatment with sarin. Data shown in Figures 6B (midbrain) and 7B (cerebellum) indicate that 3 h following treatment with sarin alone or in combination with PB resulted in significant inhibition in the enzyme activity, and pretreatment with 15 daily oral doses of PB provided some degree of protection in sarin inhibitable enzyme activity. However, the enzyme activity in the animals coexposed with sarin and PB still remained significantly inhibited (~40 and ~58% of control for midbrain and cerebellum, respectively) compared with controls.

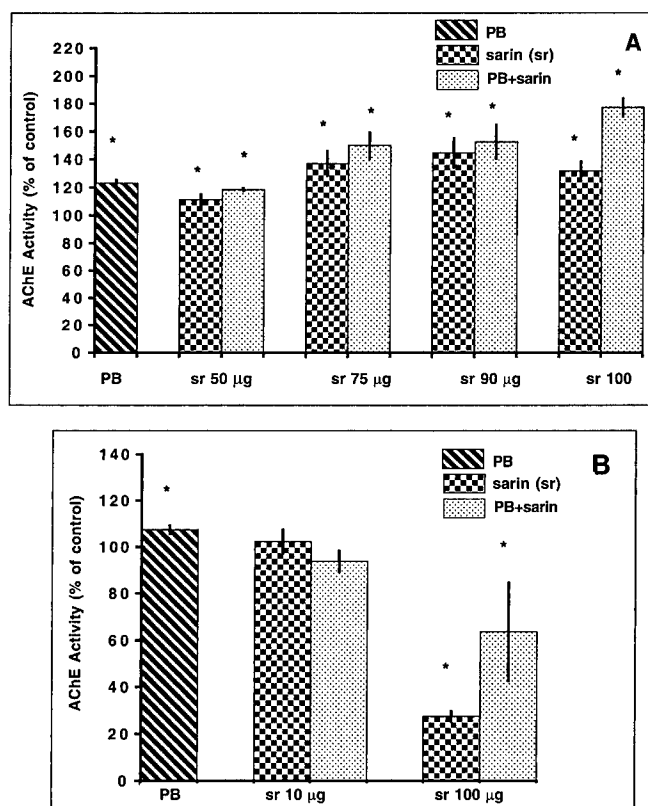


FIG. 5. Effect of treatment with sarin and PB, alone or in combination, on brain stem AChE activity. Top panel (A): The animals were pretreated with PB (1.3 mg/kg, oral, daily for 15 days) and various doses of single im injection of sarin (50, 75, 90, and 100 µg/kg) and sacrificed on day 15 following treatment with sarin. Bottom panel (B): The animals were pretreated with PB (1.3 mg/kg, oral, daily for 15 days) and single im injection of sarin (10 or 100 µg/kg) and sacrificed 3 h following treatment with sarin. The control activity was 40.1 ± 8.01 nmoles acetylthiocholine hydrolyzed/min/mg protein. Data are presented as mean \pm SE (% control), $n = 5$. *Indicates statistically significant.

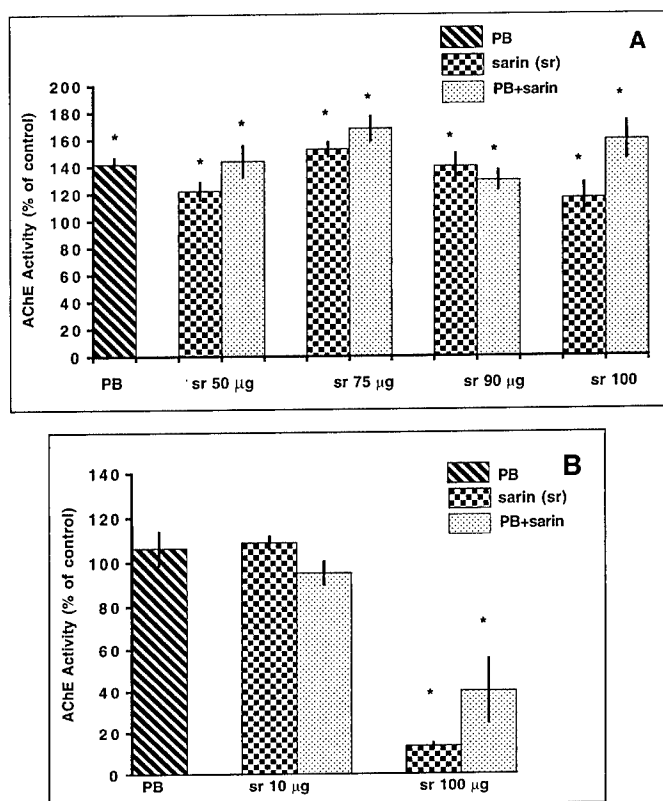


FIG. 6. Effect of treatment with sarin and PB, alone or in combination, on midbrain AChE activity. Top panel (A): The animals were pretreated with PB (1.3 mg/kg, oral, daily for 15 days) and various doses of single im injection of sarin (50, 75, 90, and 100 µg/kg) and sacrificed on day 15 following treatment with sarin. Bottom panel (B): The animals were pretreated with PB (1.3 mg/kg, oral, daily for 15 days) and single im injection of sarin (10 or 100 µg/kg) and sacrificed 3 h following treatment with sarin. The control activity was 53.8 ± 2.5 nmoles acetylthiocholine hydrolyzed/min/mg protein. Data are presented as mean \pm SE (% control), $n = 5$. *Indicates statistically significant.

Effect of Treatment with Sarin and PB, Alone or in Combination, on m_2 Muscarinic Acetylcholine Receptors in the Cortex and Brain Stem

In the animals coexposed to sarin and PB, there was a significant increase in ligand binding at 90 and 100 µg sarin dose (Fig. 8A). There was a significant increase in cortical ligand binding at 3 h following treatment with 10 µg/kg sarin and a nonsignificant increase at 100 µg/kg sarin (Fig. 8B). A similar significant increase was observed following treatment with PB alone. Brain stem ligand binding from the animals treated with 50, 75, 90, and 100 µg/kg sarin, alone or in combination with 15 days of oral pretreatment with PB, are shown Figure 9A. There was no significant effect of sarin treatment alone, whereas coexposure with sarin PB resulted in a significant increase in ligand binding at 75, 90, and 100 µg/kg sarin doses. There was no change in brain stem ligand binding at 3 h following treatment with 10 or 100 µg/kg sarin with or without treatment with PB (Fig. 9B).

DISCUSSION

These results suggest that both PB treatment alone for 15 days at a physiologically relevant route and dose and coexposure to sarin and PB caused significant sensorimotor deficits. Pretreatment with PB may afford a moderate level of protection in peripheral as well as central nervous system AChE following acute exposure with 100 µg/kg sarin. Interestingly, PB treatment alone caused significant increases in plasma BChE and brain region AChE activities long after the treatment was discontinued. The dose of PB (1.3 mg/kg) and the route of exposure in the current experiments was chosen based on the information provided by the U.S. Department of Defense to approximate the exposure conditions with PB during the PGW. Sarin dose range (50–100 µg/kg) was used to evaluate whether PB treatment would afford protection in neurobehavioral deficits, as organophosphate nerve agents are known to exhibit extended neurological deficits (Baile *et al.*, 2001; Blick *et al.*, 1991; Duffy and Burchfiel, 1980). The changes in neurochem-

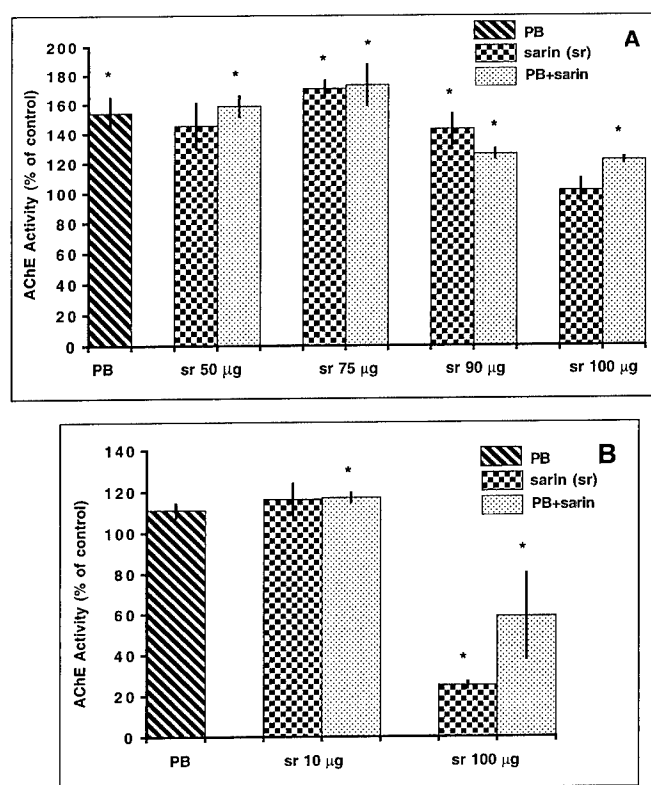


FIG. 7. Effect of treatment with sarin and PB, alone or in combination, on cerebellum AChE activity. Top panel (A): The animals were pretreated with PB (1.3 mg/kg, oral, daily for 15 days) and various doses of single im injection of sarin (50, 75, 90, and 100 µg/kg) and sacrificed on day 15 following treatment with sarin. Bottom panel (B): The animals were pretreated with PB (1.3 mg/kg, oral, daily for 15 days) and single im injection of sarin (10 or 100 µg/kg) and sacrificed 3 h following treatment with sarin. The control activity was 9.9 ± 0.62 nmoles acetylthiocholine hydrolyzed/min/mg protein. Data are presented as mean \pm SE (% control), $n = 5$. *Indicates statistically significant.

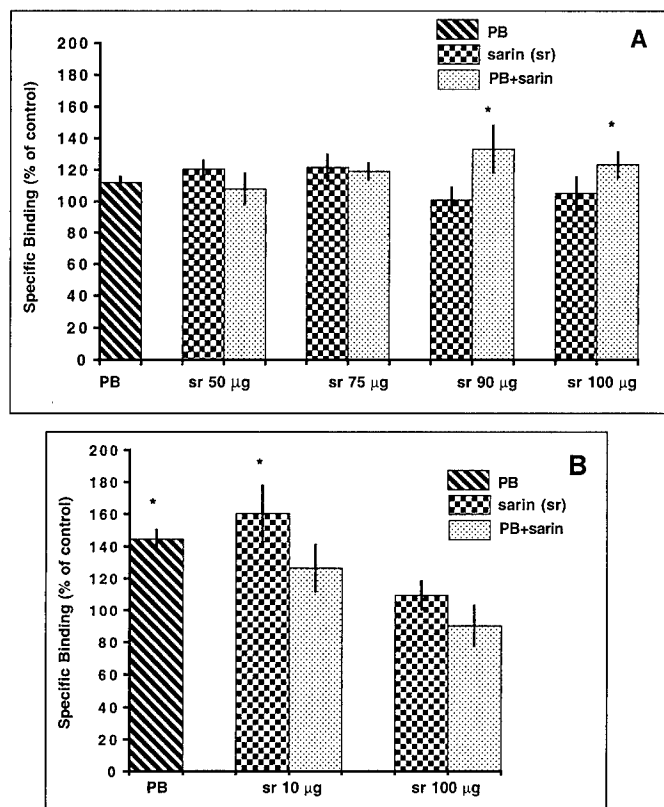


FIG. 8. Effect of treatment with sarin and PB, alone or in combination, on m2 mAChR ligand binding in the cortex. Top panel (A): The animals were pretreated with PB (1.3 mg/kg, oral, daily for 15 days) and various doses of single im injection of sarin (50, 75, 90, and 100 µg/kg, respectively, as indicated on x-axis) and sacrificed on day 15 following treatment with sarin. Bottom panel (B): The animals were pretreated with PB (1.3 mg/kg, oral, daily for 15 days) and single im injection of sarin (10 or 100 µg/kg, respectively, as indicated on x-axis) and sacrificed 3 h following treatment with sarin. The control [³H]AFDX 384 binding was 3.26 ± 0.27 f moles/min/mg protein. Data are presented as mean \pm SE (% control), $n = 5$. *Indicates statistically significant.

ical and behavioral functions observed in our studies may be related to a direct effect of PB and sarin on the cholinergic system. However, the possibility exists that long-term effects associated with PB exposure may be due to an indirect effect on the central nervous system.

Neurobehavioral data show that each treatment (PB, sarin, and PB + sarin) resulted in significant sensorimotor impairments compared with controls, which were reflected in incline plan performance, forepaw grip time, beam-walk scores, and beam-walk times at each dose and at each time point (Figs. 1 and 2). This was true even for the lowest dose of sarin used in the experiments (50 µg). As a result, it was difficult to detect a consistent effect of increasing doses of sarin when given either alone or in combination with PB (Figs. 1 and 2), although dose effects were found for at least some of the tested behavioral parameters at either of the two time points.

The control rats had stable or improving performance over the

two testing sessions. Because the sensorimotor impairments at the time of the first assessments at 7 days were severe, it was difficult to detect any further worsening over time. This worsening was significant for only some behavioral parameters.

The effect of combination PB + sarin on sensorimotor performance did not result in change compared with PB alone, suggesting little effect on sensorimotor performance of sarin when given in combination with PB. However, the combination of PB + sarin caused exacerbated effect compared with sarin alone for inclined plane performance on both days 7 and 15 and for forepaw grip on day 7, suggesting that PB added to the impact of sarin on these behavioral parameters. When these treatment groups were compared as a function of sarin dose, poorer behavioral performance was most evident only for the highest dose of sarin. In fact, for beam-walk score, PB in combination with lower doses of sarin resulted in relatively improved performance, suggesting some protective effect of the combination at lower dose. The reasons for this effect are

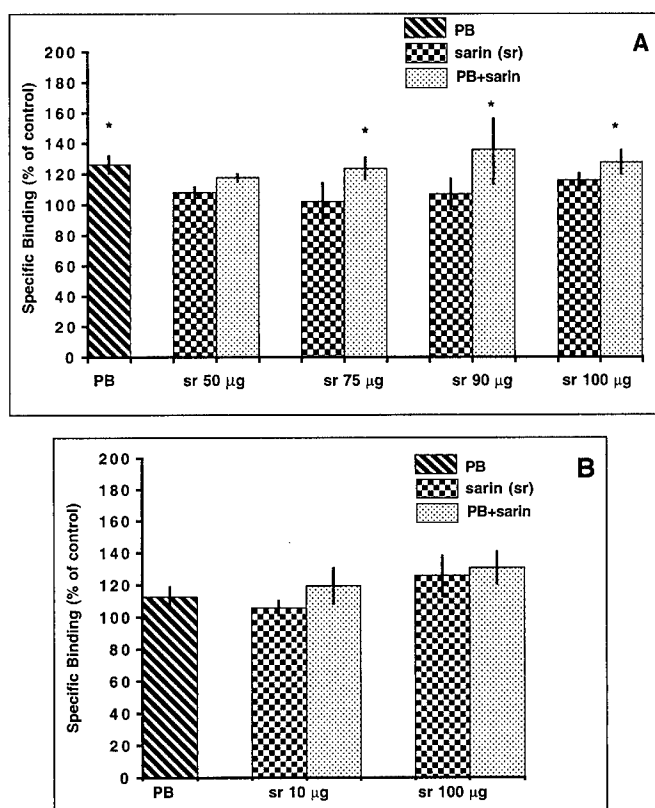


FIG. 9. Effect of treatment with sarin and PB, alone or in combination, on m2 mAChR ligand binding in the brain stem. Top panel (A): The animals were pretreated with PB (1.3 mg/kg, oral, daily for 15 days) and various doses of single im injection of sarin (50, 75, 90, and 100 µg/kg) and sacrificed on day 15 following treatment with sarin. Bottom panel (B): The animals were pretreated with PB (1.3 mg/kg, oral, daily for 15 days) and single im injection of sarin (10 or 100 µg/kg) and sacrificed 3 h following treatment with sarin. The control [³H]AFDX 384 binding was 1.65 ± 0.22 f moles/min/mg protein. Data are presented as mean \pm SE (% control), $n = 5$. *Indicates statistically significant.

unknown. Thus, these data suggest the possibility of sensorimotor deficits in the veterans of PGW, who may have been exposed to PB or sarin. These results are consistent with the epidemiological studies reported by Knoke *et al.* (2000) and Storzbach *et al.* (2000).

Pretreatment with PB for 15 days afforded mild protection in sarin-inhibited plasma BChE at 3 h of treatment, whereas 15 days after the cessation of treatment this inhibitory potential of PB diminished (Figs. 4A and 4B). Instead, there was a significant increase in plasma activity, suggesting that PB might be affecting the BChE biosynthesis in liver, the main source of the secreted enzyme in plasma (Chambers and Carr, 1993). Because PB does not readily cross the blood-brain barrier under normal conditions, it is believed that PB could not inhibit CNS AChE activity unless BBB permeability is compromised. Our data suggest that PB treatment for 15 days could cause an increase in AChE activities in cortex, brain stem, midbrain, and cerebellum. Although not universally accepted, an increase in AChE protein may reflect an increased axonal repair and synaptic modeling, as has been shown recently (Bigbee *et al.*, 2000; Guizzetti *et al.*, 1996; Sternfeld *et al.*, 1998). Our data are consistent with a recent finding by Servatius *et al.* (1998), which reported that Wistar-Kyoto (WKY) rats exhibited persistently exaggerated startle response following treatment with PB, suggesting that PB-associated neurotoxicity may have a central nervous system component. Therefore, it is possible sarin and PB treatment alone may cause subtle changes that are reflected in increased synaptic modeling and repair.

Our results also suggest that there are regional differences in the brain severity to inhibition of AChE by various doses of sarin, in that the cortical activity (Fig 4A and 4B) remained significantly inhibited for 15 days following treatment with 50, 75, 90, and 100 $\mu\text{g/kg}$ sarin, alone or in combination with PB, whereas the activity recovered in brain stem, midbrain, and cerebellum (Figs. 5A–7A). From the acute exposure data it is clear that sarin inhibition of AChE showed dose response at 10 and 100 $\mu\text{g/kg}$ sarin, whereas there was no apparent dose response at 50, 75, 90, and 100 $\mu\text{g/kg}$ sarin after 15 days. A possible explanation for this differential response may be because of “survival effect,” in that the evaluation was carried out only in the surviving animals and therefore the most severely affected at 90 or 100 $\mu\text{g/kg}$ may not have survived. In the case of brain stem, there was a significant increase in the enzyme activity at all the doses. This implies that long-term consequences of exposure to sarin alone or in combination with PB may be region specific. Our results on the effects of PB on the CNS are at variance from those reported by Friedman *et al.* (1996) in that we did not detect an inhibition in brain region AChE activity. It is possible that under stress, passage of PB would cause a direct inhibition of AChE, whereas under normal circumstances, such as under our experimental conditions, the effects of PB on the brain region AChE are mediated by indirect mechanisms. However, whether PB could have a direct

access to CNS AChE is still debatable (Grauer *et al.*, 2000; Lallement *et al.*, 1998; Sinton *et al.*, 2000).

Treatment with muscarinic antagonists induces receptor up-regulation (Ben-Barak and Dudai, 1980; Coccini *et al.*, 2000; Majocha and Baldessarini, 1984; Smiley *et al.*, 1998). Wang *et al.* (1996) reported an increase in muscarinic receptor ligand binding by repeated treatment with nicotine. Increased m2 AChR receptor ligand binding density in the brain stem in response to treatment with PB alone and in combination with 75, 90, and 100 $\mu\text{g/kg}$ sarin could reflect a compensatory mechanism for a reduced ability of these receptors to bind their respective ligands due to desensitization. The increase in ligand binding densities for m2 AChR in the brain stem may be related to the changes in the AChE levels in the cortex and brain stem following treatment with sarin and PB, alone or in combination, because of depletion of acetylcholine pool. Recently we showed that acute exposure with sarin alone at various doses differentially modulates the m2 mAChR ligand binding (Khan *et al.*, 2000). Studies by Ward *et al.* (1993) and Silveira *et al.* (1990) also have shown that organophosphate compounds selectively regulate m2 mAChR ligand binding. The data in the current study showing increased m2 mAChR-specific ligand binding in the brain stem following treatment with PB and in combination with various doses of sarin could regulate the ligand binding *in vivo*. Previously, Chaudhuri *et al.* (1993) and Liu and Pope (1996) reported an increased m2 mAChR ligand binding in response to chlorpyrifos treatment. Other studies also demonstrate that treatment with chemicals that cause inhibition of AChE lead to m2 mAChR upregulation (Majocha and Baldessarini, 1984; Nostrandt *et al.*, 1997; Witt-Enderby *et al.*, 1995). Increased ligand binding for m2 muscarinic receptor results in the inhibition of adenylate cyclase activity through a pertussis toxin-sensitive G-protein resulting in an inhibitory postsynaptic response (Brann *et al.*, 1993; Wess, 1996). The inhibitory nature of the m2 receptor may have regulatory response on GABAergic system in the cortex. It is known that cholinergic input in certain brain regions tonically inhibits the GABAergic system that it is inhibitory to vasomotor glutamergic neurons. Thus, an increase in m2 AChR in response to treatments with PB alone or in combination may regulate the glutamergic pathway, leading to a impaired motor response.

Presynaptic m2 mAChR has also been shown to cause changes in acetylcholine release via a feedback inhibitory mechanism (Gurantz *et al.*, 1993; Marchi *et al.*, 1990; Margiotta *et al.*, 1987; Raiteri *et al.*, 1984). Thus, our results suggest that the effect of sarin and PB on m2 mAChR may have modulatory effects on other processes, such as acetylcholine release and other second messenger system, that could influence the toxicity of sarin. In this context, it is noteworthy that it has been shown recently that PB induced neuronal apoptosis in rats through the activation of the muscarinic pathway (Li *et al.*, 2000). Thus, it is possible that PB exposure alone may cause CNS dysfunction by activating apoptotic pathway mediated by muscarinic receptors.

In summary, our results show significant effects of sarin and PB, alone or in combination, on sensorimotor performance as well as changes in cholinergic system in rats. Pretreatment with PB afforded protection in the PNS as well as in the CNS AChE following acute treatment with sarin, while significant neurobehavioral deficits followed 15 days of treatment with PB and sarin, alone or in combination. The anatomic and physiological mechanisms of the effects of PB and sarin on sensorimotor performance and the role of biochemical changes are uncertain and could be central, peripheral, or nonspecific. Further work is necessary in order to elucidate the mechanism of neurobehavioral deficits following exposure with PB and sarin, alone or in combination.

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Sarin Causes Altered Time Course of mRNA Expression of Alpha Tubulin in the Central Nervous System of Rats

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Sarin induced neurotoxicity is suspected to be one of the key factors responsible for Gulf-war syndrome. We studied the effect of a single (50 µg/kg/i.m) dose of sarin ($0.5 \times \text{LD}_{50}$) on the mRNA expression of alpha tubulin in the central nervous system (CNS) of rats which were sacrificed at different time points i.e. 1 and 2 hrs, as well as, 1, 3 and 7 days post-treatment. Northern data collected from CNS regions indicate differential, spatial, and temporal regulation of alpha tubulin mRNA levels. Immediate induction and persistence of alpha tubulin transcripts in sarin-treated CNS suggest that sarin-induced neurotoxicity is in part mediated by the altered expression of cytoskeletal genes which may be regulated at multiple levels.

KEY WORDS: Alpha tubulin; mRNA expression; sarin; central nervous system.

INTRODUCTION

Organophosphorous (OP) chemicals such as sarin (O-isopropylmethylphosphonofluoridate) were developed for usage as warfare agents (1). These cholinesterase inhibitors were known for their ability to inhibit the catalytic activity of the acetylcholine (ACh) hydrolyzing enzyme acetylcholinesterase (AChE). In addition to promoting immediate excitation of cholinergic neurotransmission through transient elevation of synaptic ACh levels, anti-ChEs exposure is associated with the long-term effects reminiscent of post-traumatic stress disorders (2). Several thousand soldiers were suspected to have been exposed to a combination of chemicals and possibly sarin during the Gulf War in 1990 and 1991. Some of these veterans complained of clinical symptoms that are characteristic of altered central and peripheral cholinergic systems (3).

The cytoskeleton of eukaryotic cells is one of the most important targets of neurotoxic chemicals due to the large variety of the biological events controlled by this sub-cellular structure. Microfilaments and microtubules are involved in a number of vital processes such as cell division, protoplasmic streaming, locomotion, anchorage and cellular polarity (4).

Microtubules are present as long polymers formed by the energy-dependent assembly of heterodimer subunits, composed of alpha and beta tubulins (5). The alpha and beta tubulin proteins are encoded by separate genes and exists as multiple isotypes in cells (5). Studies have shown differential mRNA expression as well as distinct assembly kinetics and ligand binding properties for different tubulin subtypes, indicating the importance of regulation at many levels (6).

We have shown that sarin-induced neurotoxicity acts through multiple mechanisms (3,7). We found earlier that diisopropylphosphorofluoridate (DFP),

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Abbreviations: ACh, Acetylcholine; AChE, Acetylcholinesterase; ATCC, American Type Culture Collection; CNS, Central Nervous System; DFP, Diisopropylphosphorofluoridate; GFAP, Glial Fibrillary Acetic Protein; Sarin; o'-isopropylmethylphosphonofluoridate; OP, Organophosphates.

which is a chemical structure analog to sarin, causes altered alpha tubulin mRNA expression in the central nervous system (CNS) of hens (8). We have also demonstrated the important role of microtubular pathology in mediating DFP-induced delayed neurotoxicity, which involves multiple mechanisms at multiple levels such as tubulin polymerization (9); altered axonal transport (10), altered phosphorylation and binding of proteins to microtubules (11) and persistent overexpression (8). The complexity of OPs induced CNS effects can be understood by the delayed and permanent nature of neurological sequel even after low-level exposures (12). Tubulin content of the brain is high, (20%) indicating its special role in the physiology and pathology of the nervous system (13). The abundant amount of tubulin in CNS cell types and the role of microtubules in axonal transport are thought to contribute to the neurologic toxicity of several hazardous chemicals (14). Since Alpha tubulin is one of the major cytoskeletal genes, shown to be altered by a variety of toxic stimuli, including organophosphates (8), studying the time course of mRNA expression of alpha tubulin is important and will elucidate the specificity of sarin action on the cytoskeleton in general and on microtubules in particular.

EXPERIMENTAL PROCEDURE

Materials. Sarin was obtained from U.S. Army Medical Research and Material Command, Fort Detrick, Frederick, MD. Alpha³²P dATP (3000 ci/mmol) was purchased from New England Nuclear (Boston, MA). Duralon-UV membranes were purchased from Stratagen (La Jolla, CA) and Random Primer Labeling system was obtained from Life Technologies, Inc (Gaithersburg, MD). Other chemicals were purchased from standard sources.

Animal Treatment. Young adult male Long-Evan rats, were purchased from Charles River, Raleigh, NC. Rats were kept in the vivarium in temperature-controlled at 21–23°C with a 12-hour light-dark cycle. All aspects of animal care, treatments and procedures were approved by the Duke University Institutional Animal Care and Use committee and to the recommended guidelines by the Army. Groups of five rats were treated with either a single intramuscular injection of 50 µg/kg/ml of sarin in normal saline or saline alone into the thigh muscle, for different treatment and control groups respectively. The rats were examined daily for any clinical signs and weighed two times a week. The rats were sacrificed at 1, 2 hrs as well as 1, 3 and 7 days post sarin-treatment. The CNS regions (i.e.; cerebrum, cerebellum, brainstem, midbrain, and spinal cord) were quickly dissected at the end of each time point and frozen in liquid nitrogen. The dissection strategy involves removal of cortex layers and cerebellum, followed by slicing of brainstem, leaving the midbrain consisting of part of striatum and hypothalamus. The tissues were kept at –70°C until used for total RNA extraction.

Preparation of cDNA Probes. Alpha tubulin and 28S RNA cDNA probes were purchased from ATCC (American Type Culture

Collection, Rockville, MD). The clones were amplified and cDNA was purified by standard methods (15). The cDNA inserts for hybridizations were prepared by Eco RI digestion and gel purified using standard protocols (15). Total RNA was purified using Trizol kit from (Life Technologies Inc, Gaithersburg, MD). Twenty micrograms of total RNA from each tissue was used for agarose gel electrophoresis and transferred to Duralon Nylon membranes. Total RNA from rats belonging to both control and treatment groups were not pooled. They were run separately in individual lanes to get radioactive measurements from each of the rats for statistical analysis. Equal loading and efficiency of transfer were verified by ethidium bromide stained gel pictures. The membranes were hybridized with radiolabeled cDNA probes. The blots were exposed to phosphorimaging plates and radioactivity in the bands was quantified with Image-quant System (Molecular Dynamics, Inc; Sunnyvale, CA). Radioactivity (PSL values) of mRNA bands from each tissue for each time point (1 hr to 7 days) was calculated as the percentage of control values. The same blot was used to hybridize both of the probes, the blot was stripped of the probe subsequently after each hybridization using 0.1% SDS and 0.1XSSC solution.

Statistical Analysis. The hybridized blot was exposed to phosphorimaging plates at least two times and the radioactivity of the band was quantified and normalized as described above. The results were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test. P value < 0.05 was considered significant. Student t-test was done to verify the significance among individual groups of the different treatments. Residuals from each tissue's ANOVA was examined using Quantile-Quantile-plots to verify the normality assumption. All the plots were consistent with data drawn from a normal population.

RESULTS

Clinical Signs. All animals were observed for the development of clinical signs of toxicity. All animals treated with 0.5 × LD₅₀ developed tremors by 15–30 minutes after treatment. There were no observable changes in clinical signs thereafter.

mRNA Expression of Alpha Tubulin in the Cortex of Sarin-Treated Rats. Sarin-treated rats showed a small but significant increase in alpha tubulin transcript levels at 1 hr (127 ± 2%) and the levels further increased to 142 ± 3% at 2 hrs. At 1 day (141 ± 4%) and 7 days (137 ± 8%) the levels stayed more or less the same, with a slight dip in the intervening time-point at 3 days (121 ± 6%) (Fig 1, 2A). The expression of 28S RNA was used as control to reveal any variation in alpha tubulin expression due to purity of RNA or error in estimation and loading. The same northern blot was hybridized to both probes and the blot was stripped after hybridization with each probe. The 28S RNA did not show any significant change in their expression at any time point after sarin treatment. Alteration in the expression of alpha tubulin was real and could not be ascribed to the above mentioned

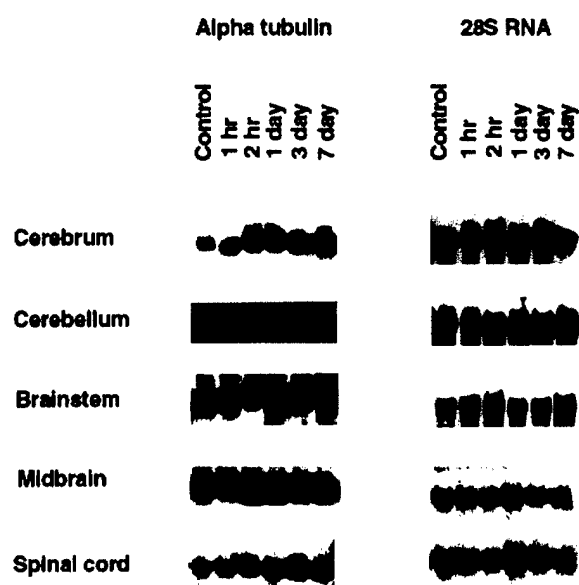


Fig. 1. Northern blots showing the expression of Alpha tubulin and 28S RNA in the central nervous system of sarin-treated rats. Rats were treated with a single dose of sarin ($0.5 \times \text{LD}_{50}$) and sacrificed at 1 and 2 hrs, as well as 1, 3, and 7 days. Total RNA ($20 \mu\text{g}$) from cerebrum, cerebellum, brainstem, midbrain and Spinal Cord were used for northern blotting as described in materials and methods. The blots were stripped off and used for the next one each time. This figure shows only the relevant portion of representative autoradiograms, although five rats were used in each treatment group. There were significant alterations for alpha tubulin in all the tissues studied, indicating temporal, spatial, and differential expression pattern.

errors, since alpha tubulin mRNA showed a time-course profile different from that shown by the control message. The mRNA expression data of alpha tubulin was normalized to control 28S RNA in all the tissues.

mRNA Expression of Alpha Tubulin in the Cerebellum of Sarin-Treated Rats. A single dose of sarin ($0.5 \times \text{LD}_{50}$) showed a significant induction of alpha tubulin to $145 \pm 9\%$ at 1 hr and the levels were down-regulated to $117 \pm 5\%$ at 2 hrs. However the transcript levels reached a peak to $225 \pm 3\%$ at 1 day. There was another dip in the transcript level to $131 \pm 4\%$ at 3 days and the levels raised again to $169 \pm 4\%$ at 7 days (Fig 2B). The 28S RNA did not show any significant change in their expression at any time point after sarin treatment (Fig 1).

mRNA Expression of Alpha Tubulin in the Brainstem of the Sarin-Treated Rats. Sarin treated rats did not show any statistically significant differences from the control rats in the levels of alpha tubulin transcripts until day 1. The levels were induced to $124 \pm 7\%$ at 1 day and they further increased to $142 \pm 5\%$ at

3 days. The peak level of $246 \pm 9\%$ was noted at 7 days (Fig 2C). The 28S RNA did not show any significant change in their expression at any time after sarin treatment (Fig 1).

mRNA Expression of Alpha Tubulin in the Mid-Brain of Sarin-Treated Rats: Sarin-treated rats showed a small but significant increase in alpha tubulin transcript levels at 1 hr to $151 \pm 14\%$. After an initial down-regulation to $132 \pm 6\%$ at 2 hrs, the levels remained more or less in the same range at 1 day (134 ± 15), 3 days ($118 \pm 4\%$) and 7 days ($136 \pm 6\%$) (Fig 2D). The 28S RNA did not show any significant change in their expression at any time point after sarin treatment (Fig 1).

mRNA Expression of Alpha Tubulin in the Spinal Cord of Sarin-Treated Rats: A single dose of sarin ($0.5 \times \text{LD}_{50}$) showed a significant induction of alpha tubulin at 1 and 2 hrs to 134 ± 2 and $156 \pm 6\%$ respectively. The levels further increased to $242 \pm 19\%$ at 1 day. There was a further increase in the transcript levels at 3 days to $288 \pm 11\%$. At 7 days the transcript levels showed a downward trend to $136 \pm 7\%$ (Fig 2E). The 28S RNA did not show any significant change in their expression at any time point after sarin treatment (Fig 1).

DISCUSSION

We have found altered expression of alpha tubulin mRNA showing differential, spatial, and temporal pattern in sarin treated CNS of the rats, which may be one of the important factors involved in the molecular pathogenesis related to sarin-induced toxicity. This data is consistent with our hypothesis that organophosphate-induced neurotoxicity involves changes in cytoskeleton at multiple levels [16]; (8)]. Increased alpha tubulin levels has been shown to be involved in neurites extension, axonal regeneration (16), and axonal degeneration (8). Increased sensitivity by astroglial cells, as evidenced by altered mRNA expression of GFAP (Glial Fibrillary Acetic Protein) and vimentin (unpublished data), may have an additive effect on modified levels of alpha tubulin (8).

Differential mRNA expression of alpha tubulin can be attributed to the changes in polymerization, phosphorylation, transport of tubulins and protein expression, similar to our earlier findings with DFP-treatment (8). These results support the fact that functional and regional differences of the CNS could be contributing to the differential response to sarin. The differential expression pattern observed could be due to

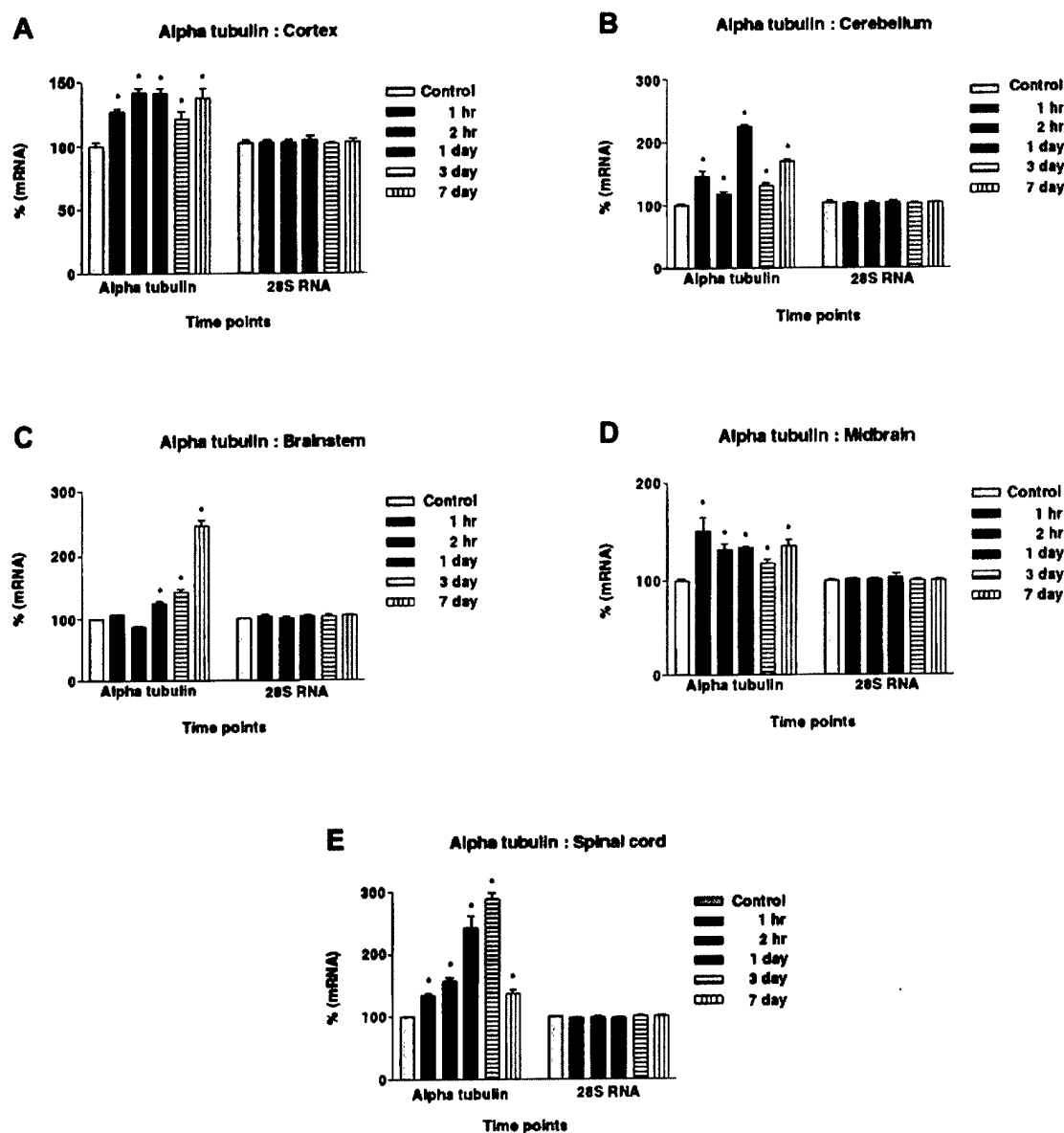


Fig. 2. Expression of alpha tubulin in sarin-treated rat CNS tissues as compared to controls: Rats were treated with a single dose of sarin ($0.5 \times LD_{50}$) and sacrificed at 1 and 2 hrs, as well as 1, 3, and 7 days. Total RNA was purified from the CNS tissues of each rat and 20 μ g was used for the northern blotting using standard techniques and hybridization was carried out as described in materials and methods. Profiles of mRNA expression represent the means \pm SEM of the percent of control values from untreated rats. The radioactivity of bands was measured in PSL units by exposing northern blots to phospho-imaging plates, followed by quantification with the Fuji Bio-Imaging system. Five rats were used in each treated group and the controls. Significant difference ($P < 0.05$) from the control is marked with an asterisk. Sarin-treated CNS tissues showed distinct differential, spatial, and temporal expression pattern. No significant changes were seen for 28S RNA at any time point.

the differential susceptibility as well as due to the extensive heterogeneity of the brain tubulin content (8).

The immediate induced levels of alpha tubulin noticed in different CNS regions ranged from small (cortex) to high (midbrain) except brainstem, where there was no significant level of induction in early

time points. The brainstem, however has been identified as a more responsive region of the CNS to sarin-treatment (3). Absence of induction in the brainstem during early time points can be attributed to toxicant induced trauma. However, brainstem is also the only tissue that showed steady time-dependent increase in

alpha-tubulin levels and reached peak levels at 7 days. This probably supports the idea that the initial trauma was followed by the induction of recovery or degenerating processes at later time points (17). The other more-responsive region, midbrain, showed immediate induction and persistence of alpha tubulin levels. Thus, local microenvironment plays an important role in sarin-induced neurotoxicity, along with degree of susceptibility and tubulin isotype variability similar to our earlier findings on DFP-induced neurotoxicity (8).

The cerebellum is the only tissue that showed great degree of fluctuations in the transcript levels. This may be a result of the complexity of tissue architecture due to cellular heterogeneity and specialized cells types (8). The cortex showed more or less the same moderately induced levels for most of the time course. This expression pattern was different from that of other cytoskeletal genes such as GFAP and vimentin, which showed early higher induction and overall downward trend at later time points (unpublished). Spinal cord on the other hand, showed an expression pattern similar to GFAP and vimentin (unpublished). Altogether our findings support the notion that tubulins are regulated at multiple levels (8). Sarin-induced changes in the rate of tubulin polymerization resulting in changed levels of free tubulin monomers may be involved in the altered alpha tubulin mRNA expression at different time points in different tissues by auto-regulatory circuits, similar to our findings in hens treated with DFP (8).

This study suggests that sarin treatment caused alteration of different genes through distinctly different pathways. Immediate induction and persistence of alpha tubulin transcript levels in most tissues of the CNS strengthen the hypothesis that sarin-induced neurotoxicity is in part mediated by the altered expression of cytoskeletal genes, which may be regulated at multiple levels.

ACKNOWLEDGMENTS

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Sarin Causes Early Differential Alteration and Persistent Overexpression in mRNAs Coding for Glial Fibrillary Acidic Protein (GFAP) and Vimentin Genes in the Central Nervous System of Rats

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Neurotoxic effects of single dose of $0.5 \times LD_{50}$ sarin (*O*-isopropylmethylphosphonofluoridate) on central nervous system (CNS) of male Sprague-Dawley rats were studied. We investigated the mRNA expression of the astroglial marker genes glial fibrillary acidic protein (GFAP) and vimentin to evaluate the fate of astroglial and neuronal cells, because reactive gliosis is very often used to assess the extent of CNS damage. Rats were treated with 50 $\mu\text{g/kg/ml}$ of sarin and terminated at the time-points 1 and 2 hours and 1, 3, and 7 days post-treatment. Control rats were treated with normal saline. Total RNA was extracted and Northern blots were hybridized with cDNA probes for GFAP and vimentin, as well as 28S RNA (control). The data obtained indicate that a single dose of sarin ($0.5 \times LD_{50}$) showed induction in the transcript levels of GFAP and vimentin in the cortex, cerebellum, brainstem and midbrain, and spinal cord. The induction showed distinct spatial-temporal differences for each tissue studied. Both GFAP and vimentin were induced at 1 hour in all the tissues studied except brainstem, where moderate and high levels of GFAP induction were noted at 1 and 3 days. Overexpressed transcript levels of GFAP and vimentin remained high in more responsive tissues such as the brainstem and midbrain. Other tissues, such as the cortex, spinal cord, and cerebellum showed a more downward trend for either GFAP or vimentin, or both, transcript levels at 7 days. It is noteworthy that both cortex ($318 \pm 12\%$) and spinal cord ($368 \pm 12\%$) showed relatively higher induction of GFAP, whereas cortex alone showed the highest level of overexpressed vimentin transcript levels ($284 \pm 11\%$). Overall it is also clear that both GFAP and vimentin are needed for the effective recovery involving co-ordinated alternating up- and down-regulation of these two key astrocyte genes, depending on tissue specificity. The changes seen in the transcript levels of GFAP and vimentin may be the result of astrocyte dysfunction and loss, accompanied by compensatory proliferation and dedifferentiation of the astroglia. These changes could affect the neuronal cell types, thus altering the neuron-glia homeostasis.

KEY WORDS: GFAP; vimentin; central nervous system of rat; sarin; differential expression.

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Abbreviations: AChE, acetylcholinesterase; ATCC, American Type Culture Collection; AP-1, activated complex protein-1; BBB, blood-brain barrier; BG, Bergmann glia; CREB, cAMP-response element binding protein; CNS, central nervous system; CTC, cytotoxic T lymphocytes; DEPC, diethylpyrocarbonate; DFP, diisopropylphosphorofluoridate; DMSO, dimethyl sulfoxide; GFAP, glial fibrillary acidic protein; IEG, immediate early gene; IL-1, interleukin-1; IL-6, interleukin-6; IF, intermediate filament; JNK, *c-jun*-N-terminal kinase; m2-mAChR, m2-selective muscarinic acetylcholine receptor; MAPK, mitogen activated protein kinase; NK, natural killer; OPIDN, organophosphorus ester-induced delayed neurotoxicity; PNS, peripheral nervous system; PC, Purkinje cells; SCE, sister chromatid exchange; TGFB-3, transforming growth factor beta; TNF-alpha, tumor necrosis factor alpha.

INTRODUCTION

Organophosphorus-esters have been widely used in industry, household items, medicines, and agricultural products (1). Some organophosphorus-esters, such as sarin (*O*-isopropylmethylphosphonofluoridate), were developed as warfare agents (2). These chemicals were known for their ability to inhibit the catalytic activity of the acetylcholine (ACh) hydrolyzing enzyme acetylcholinesterase (AChE). This results in the promotion of immediate excitation of cholinergic neurotransmission through transient elevation of synaptic ACh levels, and the anti-ChEs exposure is associated with the long-term effects reminiscent of post-traumatic stress disorders (3). Several thousand American soldiers were suspected to be exposed to a combination of chemicals and possibly sarin during the Gulf War. Some of these veterans complained of clinical symptoms characteristic of altered central and peripheral cholinergic system (4).

Reactive gliosis is the most prominent response to diverse forms of central nervous system injury. The signaling events that mediate this characteristic response to neuronal injury have been shown to be due to the activation of phosphoproteins. Sarin-like compounds have been shown increase the levels of tyrosine phosphorylation of several proteins in the cytosol fraction of the rat brain and activation of *c-jun*-N-terminal kinase (JNK) and mitogen activated protein kinase (MAPK) in the cytosol (5). These signaling pathways may be important and responsible for the glial response following injury, by virtue of their ability to phosphorylate and regulate the activity of various transcription factors.

Central nervous system (CNS) neurons are surrounded by astroglial cells, and these glial cells are implicated in diverse functions such as maintenance of the blood-brain barrier (6); regulation of water, ion, and amino-acid neurotransmitter metabolism (7); energy and nutrient support of neurons (8); modulation of immune/inflammatory responses (9); and phagocytic functions (8); neuronal migration (10); neurite outgrowth (8); and synaptogenesis and synaptic plasticity (6). Alterations in the glial environment induce changes in glial morphology and phenotype (9). Although predominantly expressed in astrocytes, glial fibrillary acidic protein (GFAP) is expressed in cells outside the CNS such as non-myelinating Schwann cells, the epithelial cells of salivary glands and their neoplasms, and neoplastic cells of mullerian origin (11). Schwann cells express a GFAP mRNA (GFAP- β) (12) that differs from the major CNS type,

mRNA (GFAP- α) by the presence of an extended 5' untranslated region (13,14).

Vimentin is initially expressed by nearly all neuronal precursors *in vivo* and is gradually replaced by neurofilament genes shortly after the immature neurons become postmitotic (15) and in astroglial cells by GFAP (16). In adult animals, vimentin antibodies stain sheath material around the brain, a monolayer of ependymal cell bodies lining the ventricles, fibrous material associated within the choroid plexus, the walls of blood vessels and capillaries, and the processes of cells in certain regions (17). Levels of GFAP and vimentin are regulated under developmental and pathological conditions. GFAP and vimentin are required for proper formation of glial scar following trauma to the CNS (18). We have demonstrated the differential modulation of mRNA levels of GFAP and vimentin in the CNS of hens treated with DFP (diisopropylphosphorofluoridate), a structural analog to sarin (19). DFP also decreased the protein level of vimentin and GFAP in the peripheral nervous system of hens during OPIDN (20).

Our laboratory has shown that a single exposure of sarin in rats, ranging from 0.1 to $1 \times \text{LD}_{50}$ modulates the cholinergic pathways differently in different regions and thereby causes dysregulation in excitatory neurotransmission, indicating that sarin-induced neurotoxicity has multiple mechanisms (4). Sub-chronic neurotoxic studies with sarin have also shown differential effect on different regions such as cortex, cerebellum, brainstem, and midbrain with reference to acetylcholinesterase inhibition, M2-mAChR ligand binding, and blood-brain barrier (BBB) permeability (21). More recently we have shown the immediate and differential induction of alpha tubulin, one of the major cytoskeletal genes in the CNS of sarin-treated rats (22). Increased sister chromatid exchanges (SCE) in sarin-exposed humans (23) and decreased natural killer (NK) and cytotoxic T lymphocyte (CTL) activity in sarin-treated mice (23) (24) have been reported. Altered DNA and protein metabolism has been reported (25). Murata et al. (26) concluded that sarin may have neurotoxic effects independent of brain ChE inhibition. Thus, targets of sarin action may be very complex, involving multiple pathways and resulting in regulation at multiple levels. Hence, we investigated the fate and response of astroglial cells to sarin treatment by studying the time course of mRNA expression of two main astroglial marker genes, GFAP and vimentin, which might give more insight on the development of sarin-induced neurological syndromes.

EXPERIMENTAL PROCEDURE

Materials. Sarin was obtained from U.S. Army Medical Research and Materiel Command, Fort Detrich, Frederick, MD. Radioactive (α - 32 P)dATP (3000 ci/mmol) was purchased from New England Nuclear (Boston, MA). Duralon-UV membranes were purchased from Stratagen (La Jolla, CA), and the Random Primer labeling system was obtained from Life Technologies, Inc. (Gaithersburg, MD). Other chemicals used were purchased from standard sources.

Animal Treatment. Young adult male Long-Evan rats of known age, weighing approximately 250 grams were purchased from Charles River, Raleigh, NC. Rats were kept in the vivarium with temperature controlled at 21–23°C, with a 12-hour light-dark cycle. They were provided with Rat Chow (Purina, St. Louis, MO) and tap water ad libitum. Animal treatment with sarin was carried out in a specially protected treatment room at Duke University. All animal treatments and procedures were approved by the Duke University Institutional Animal Care and Use Committee and according to the U.S. Army recommended guidelines. All efforts were made to minimize animal suffering, to use only the number of animals necessary to produce reliable scientific data, and to utilize alternatives to in vivo techniques, if available. Groups of five rats were treated with a single intramuscular injection of 50 μ g/kg/ml in normal saline into the thigh muscle, for each time point. The control group containing five rats was injected with saline as described above. The rats were examined daily for any clinical signs and weighed two times a week. At the termination of the experiment, the animals were anesthetized with 0.2-ml ketamine/xy-lazine and the animals were dissected. The brain was removed and washed thoroughly with ice-cold DEPC-treated water to remove traces of blood. The rats were sacrificed at 1 and 2 hours, as well as 1, 3, and 7 days after sarin treatment. The different brain regions (i.e., cerebrum, cerebellum, brainstem, and spinal cord) were quickly dissected at the end of each time point and frozen in liquid nitrogen. The tissues were kept at -70°C until used for total RNA extraction.

Preparation of cDNA Probes. GFAP α , vimentin, and 28S RNA cDNA probes were used for Northern blot hybridization. All the probes were purchased from ATCC (American Type Culture Collection, Rockville, MD). The clones were amplified, and cDNA was purified by standard methods (27). All the cDNA inserts for hybridization were prepared by Eco R I digestion to release the fragment from its vector and gel purified using standard protocol (27).

Northern Blot Hybridization. Total RNA was purified from cerebrum, cerebellum, brainstem, and spinal cord from control and sarin-treated rats by Trizol kit from (Gibco-BRL, Gaithersburg, MD). Twenty micrograms of total RNA from each tissue was used for agarose gel electrophoresis and transferred to Duralon Nylon membranes. The membranes were hybridized with P^{32} -labeled cDNA probes and later labeled by the Random Primer labeling system using (α - 32 P) dATP as the radioactive nucleotide. The blots were exposed to phosphorimaging plates, and radioactivity in the bands was quantified with the Imagequant System (Molecular Dynamics, Inc; Sunnyvale, CA). Radioactivity (PSL values) of mRNA bands from each tissue for each time point (1 hour to 7 days) was calculated as the percentage of value obtained for the same tissue from control rats. The radioactivity of the mRNA bands from control tissues for any probe, and tissue was assigned as 100%. The total RNA from both the control and treated rats were not pooled, but were run separately on individual lanes in the same Northern gel. This helped us

to collect radioactive measurements of each hybridizing band, representing all the rats. The same blot was used to hybridize all three probes, the blot was stripped off the probe subsequently after each hybridization using 0.1% SDS and $0.1 \times$ SSC solution.

Statistical Analysis. The hybridized blots were exposed to phosphorimaging plates at least two times, and the radioactivity of band was quantified and normalized as described above. The result was analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test. P value < 0.05 was considered significant. The Student t-test was done to verify the significance among the individual groups of different treatments. Residuals from each tissue's ANOVA were examined using quantile-quantile plots to verify the normality assumption. All the plots were consistent with data drawn from a normal population.

RESULTS

Clinical Signs

All animals were observed for the development of clinical signs of toxicity. All animals treated with $0.5 \times \text{LD}_{50}$ developed tremors by 15–30 minutes after treatment. There were no observable changes in clinical signs thereafter.

mRNA Expression of GFAP and Vimentin in the Cortex of Sarin Treated Rats

A single dose of sarin (50 μ g/kg) produced a significant increase in GFAP transcript level, to $269 \pm 7\%$ at 1 hour, and the levels increased further to $318 \pm 12\%$ at 2 hour (Fig. 1). However, the levels were down-regulated to $131 \pm 3\%$ at 1 day, which remained more or less the same for 3 days ($135 \pm 7\%$) and at $132 \pm 5\%$ for 7 days. The levels of mRNA coding for vimentin showed a pattern consistent with alternating expression with that of GFAP. Like GFAP, vimentin also showed early induction, at 1 hour ($183 \pm 1\%$) and continued to increase at 2 hour ($236 \pm 9\%$). The peak induction was noted at 1 day ($284 \pm 11\%$). Vimentin expression continued to remain high at 3 days ($243 \pm 16\%$) and 7 days ($227 \pm 13\%$). The expression of 28S RNA was used as control to reveal any variation in GFAP and vimentin expression resulting from purity of RNA or error in estimation and loading (Fig. 1). The same Northern blot was hybridized to all three probes, and the blot was stripped after hybridization with each probe. The 28S RNA did not show any significant change in expression at any time point after sarin treatment. Alteration in the expression of GFAP and vimentin was real and could not be ascribed to the

above-mentioned errors, because both mRNAs showed a time-course profile different from that shown by the control message. The mRNA expression data of GFAP and vimentin were normalized to control 28S RNA in all the regions (Fig. 2A).

mRNA Expression of GFAP and Vimentin in the Cerebellum of Sarin-Treated Rats

Both GFAP and vimentin levels showed distinctly different expression profiles, closely resembling an alternating expression pattern. Both GFAP and vimentin showed early induction at 1 hour ($157 \pm 6\%$) and 2 hours ($135 \pm 5\%$) (Fig. 1). At 2 hours, vimentin levels were near control levels ($107 \pm 5\%$), whereas GFAP levels were at $130 \pm 3\%$. Transcript levels for GFAP remained approximately the same ($132 \pm 2\%$ and $138 \pm 5\%$ for 1 and 3 days, respectively). A dramatic increase to $180 \pm 4\%$ occurred at 7 days. Vimentin levels increased quickly to $161 \pm 4\%$ at 1 day and reached a peak at 3 days ($182 \pm 10\%$). Transcript levels for vimentin dropped back to ($126 \pm 6\%$) at 7 days (see Fig. 2B). There was no alteration in 28S RNA levels at any time point in sarin-treated cerebellum.

mRNA Expression of GFAP and Vimentin in the Midbrain of Sarin-Treated Rats

A significant increase occurred in GFAP and vimentin transcript levels ($130 \pm 6\%$ and $143 \pm 7\%$) at 1 hour (Fig. 1). Overall, from 2 hours onward; GFAP transcript levels showed higher induction in this tissue than vimentin. Vimentin levels dropped to 118 ± 4 at 2 hours and continued to remain more or less within the range at $120 \pm 5\%$, $131 \pm 4\%$, and $138 \pm 9\%$ at 1, 3, and 7 days, respectively. In contrast, GFAP transcript levels showed slight increase to $141 \pm 5\%$ at 2 hours and reached the peak level at 1 day ($191 \pm 7\%$). In spite of a slight drop in the mRNA levels at 3 days ($173 \pm 13\%$), the levels remained high, at $159 \pm 9\%$, at 7 days (Fig. 2C). There was no alteration in 28S RNA levels at any time point in sarin-treated rat CNS, compared with the controls.

mRNA Expression of GFAP and Vimentin in the Brainstem of Sarin-Treated Rats

In brainstem, vimentin showed a significant expression pattern (Fig. 1). Although GFAP transcript

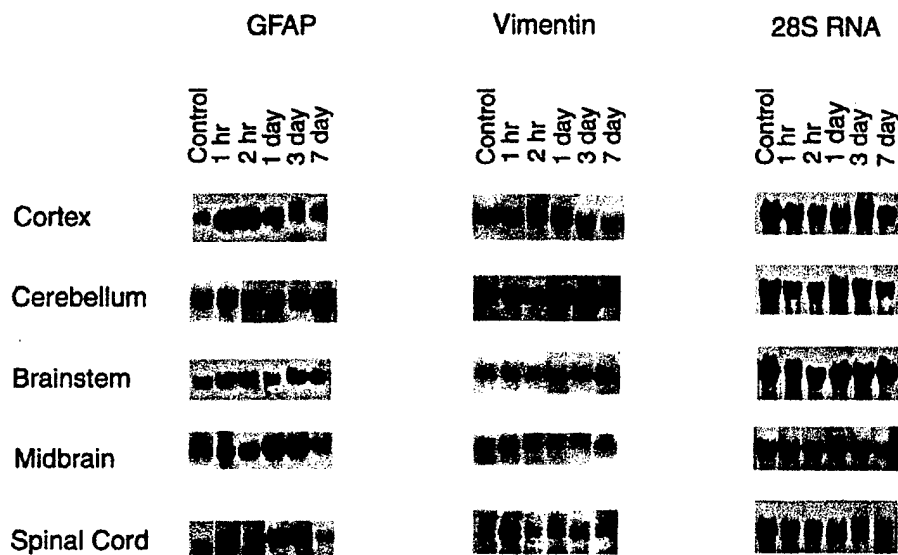


Fig. 1. Northern blots showing the expression of GFAP- α , vimentin, and 28S RNA in the CNS of sarin-treated rats. Rats were treated with a single dose ($50 \mu\text{g/kg/ml}$) of sarin and sacrificed at 1 and 2 hours and 1, 3, 7 days post-treatment. Total RNA ($20 \mu\text{g}$) from cortex, cerebellum, brainstem, midbrain, and spinal cord were used as described in Materials and Methods. The ^{32}P -labeled probes prepared from cDNA probes of GFAP- α , vimentin, and 28S RNA were used. Each hybridizing band was quantified using the Imagequant System (Molecular Dynamics, Inc; Sunnyvale, CA). Radioactivity (PSL values) of mRNA bands were calculated as the percentage of value for the same tissue from the control rats. The blots were stripped of one probe and used for the next one each time. This figure shows only the relevant portion of representative autoradiograms, although five rats were used in each treatment group. There were significant alterations for GFAP and vimentin in all the tissues studied, indicating temporal, spatial, and differential pattern.

levels remained near control levels at 1 hour ($110 \pm 2\%$), at 2 hours ($106 \pm 2\%$) and 1 day ($121 \pm 2\%$), vimentin levels increased significantly to $145 \pm 6\%$ at 1 hour and remained more or less at the same level ($141 \pm 3\%$) at 2 hours. The dramatic induction of vimentin to $189 \pm 6\%$ at 1 day, followed by a second peak to $214 \pm 9\%$ at 7 days had an intervening very slight decrease to $174 \pm 8\%$ at 3 days. GFAP transcript levels were also induced at 3 days ($214 \pm 8\%$) and 7 days ($182 \pm 9\%$) (Fig. 2D). There was no alteration in 28S RNA levels at any time point in sarin-treated brainstem ($138 \pm 9\%$). In contrast, GFAP transcript levels showed a slight increase to $141 \pm 5\%$ at 2 hours and reached the peak level at 1 day ($191 \pm 7\%$). There was a decrease in the mRNA levels at 3 days ($173 \pm 13\%$), and the levels remained high ($159 \pm 9\%$) at 7 days (Fig. 2D). There was no alteration in 28S RNA levels at any time point in sarin-treated rat CNS, compared with the controls.

mRNA Expression of GFAP and Vimentin in the Spinal Cord of Sarin-Treated Rats

In the spinal cord, early time points showed more vimentin induction, whereas GFAP levels were over-expressed during the later time points (Fig. 1). While GFAP levels increased gradually, vimentin levels remained more or less the same for the significant part of the time-course. Transcript levels of GFAP were $177 \pm 4\%$, $214 \pm 7\%$, $246 \pm 6\%$, $368 \pm 12\%$, $380 \pm 14\%$, and $134 \pm 5\%$ at 1 and 2 hours and 1, 3, and 7 days, respectively. Similarly, vimentin transcript levels were $198 \pm 8\%$, $168 \pm 10\%$, $189 \pm 6\%$, $177 \pm 11\%$, $132 \pm 4\%$, and $135 \pm 7\%$ for 1 and 2 hours and 1, 3, and 7 days, respectively (Fig. 2E). There was no alteration in 28S RNA levels at any time point in sarin-treated rat CNS, compared with the controls.

DISCUSSION

We have earlier shown that sarin-induced changes in cholinergic pathways are distinctly different for each sub-region of CNS, thereby strongly supporting the possibility of multiple mechanisms involved in sarin-induced neurotoxicity (4). Astrocytes demonstrate a multitude of responses to brain insults, including hypertrophy and often increased GFAP levels (28,29). Such astrocyte reactions relate in a dose-, time-, and region-dependent manner to the extent of nerve cell damage. Astrocytes are also believed to participate in the regula-

tion of neurotransmission. Region-specific changes in GFAP and vimentin levels in sarin-induced rat CNS add support to the idea that structural and functional differences in astroglial cells determine the fate of the different astroglial cell types (30).

The alterations in mRNA levels of GFAP and vimentin may be due to change in either the rate of transcription of DNA or the stability of GFAP and vimentin mRNA. It is hard to speculate at this time which of the following pathways may be responsible for the induction: (i) Early induction of immediate early genes (IEGs), consequently resulting in modulation of GFAP and vimentin transcription. Earlier we have shown that organophosphates such as DFP can induce IEGs such as *c-fos* and *c-jun* (31,32). Other laboratories reported similar results (33). Because sarin is very similar to DFP, one can speculate that IEGs induced by sarin may act on binding sites in the promoters of GFAP and vimentin (ii) Early induction and persistence could be the result of activation of several inflammatory cytokines, such as interleukin-6 (IL-6) (34), interleukin-1 (IL-1), (35), transforming growth factor (TGF- β) (36), and tumor necrosis factor alpha (TNF- α) family of genes (37), whose expression has been directly correlated to astroglial changes and modified mRNA expression of GFAP. The 5' upstream promoter of GFAP contains numerous *cis*-acting regulatory elements (38). GFAP expression has been shown to be the result of transcriptional activation by TGF- β -1 and repression by IL-1 (39). It was suggested that regional differences in the injury response might be due to local differences in the cytokine concentration (40). Even though the induction or suppression of mRNA levels need not necessarily increase or decrease the concentration of GFAP and vimentin proteins, it can be predicted that there may be alterations in protein levels, based on our preliminary immunohistochemistry data. Early induction, at 1 hour and gradual increase and persistence of over-expression of GFAP and vimentin until later time points, such as 3 and 7 days, in many of the tissues of current study clearly support the long-term implications of immediate early changes.

Early induction of both GFAP and vimentin has been observed in cortex, cerebellum, midbrain, and spinal cord at 1 hour to varying levels. However, there was no induction of GFAP in brainstem until 1 day, in spite of significant level of induction of vimentin at 1 hour. It is also very interesting to note that the two tissues (midbrain and brainstem) that were identified to be more responsive to sarin treatment at low doses (4) at early time points showed persistent and highly

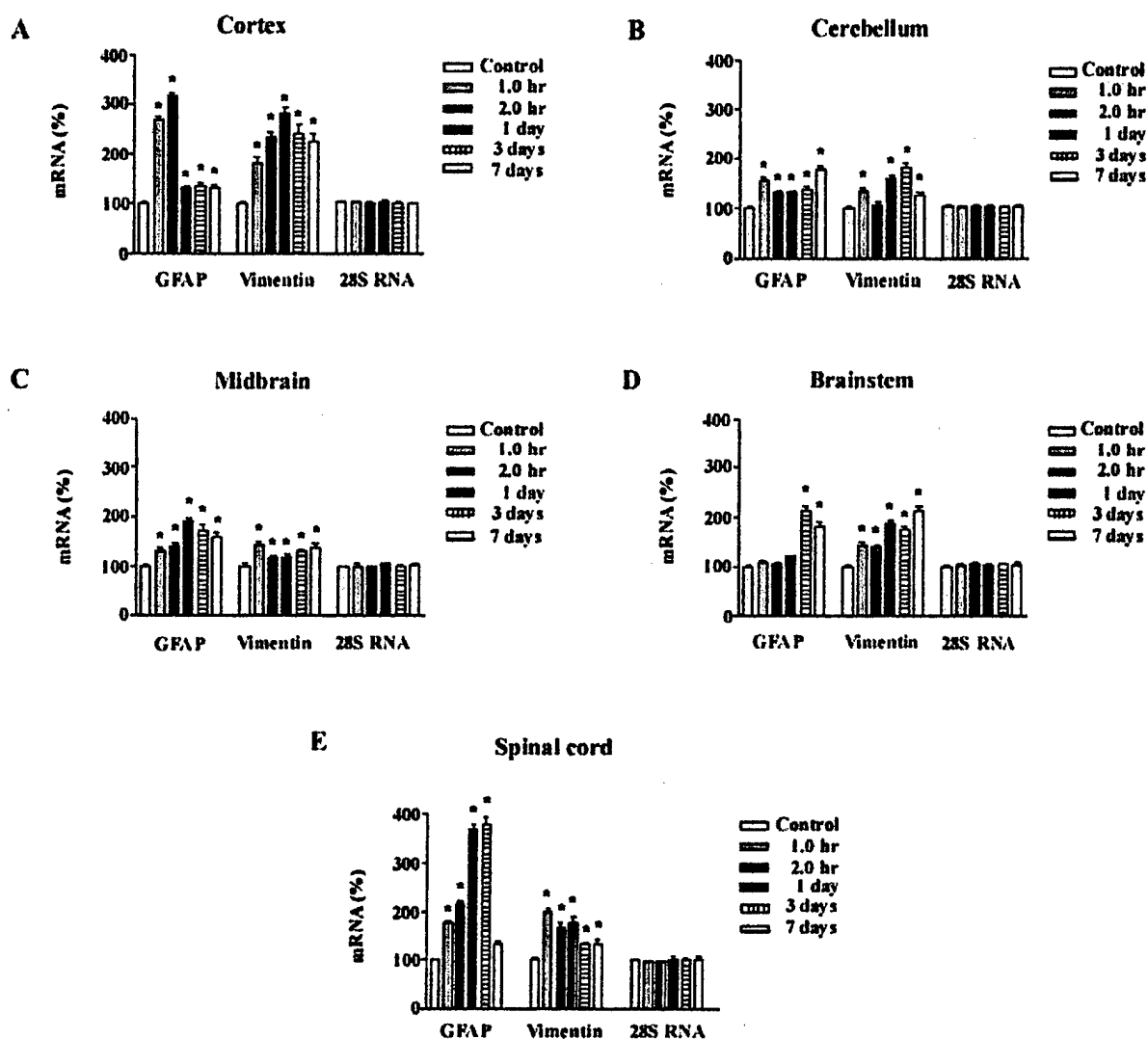


Fig. 2. Expression of GFAP- α , vimentin, and 28S RNA and mRNAs in the cortex of sarin-treated rats. Rats were treated with a single dose of sarin (50 μ g/kg/ml) and sacrificed after 1 and 2 hours and 1, 3, and 7 days after treatment. Total RNA was purified from the cortex of each rat, 20 μ g was used for Northern blotting using standard techniques, and hybridization was carried out as described in materials and methods. GFAP, vimentin, and 28S RNA cDNA probes were labeled with (alpha 32 P) dATP by the random priming method. Profiles of mRNA expression represent the mean \pm SEM of the percent of control values from untreated rats. The radioactivity of bands was measured in PSL units by exposing Northern blots to phosphorimaging plates, followed by quantification with the Fujix Bio-Imaging system. Five rats were used in each treated group, and the control group consists of 5 rats. Significant difference ($P < 0.05$) from the control is marked with an asterisk. There was a large statistically significant increase in GFAP transcript level at 1 and 2 hours and a small increase at subsequent time points. Vimentin showed significant increase at all time points. No significant change was noticed for 28S RNA at any time points for any tissues studied, and hence the GFAP and vimentin data have been normalized to 28S RNA data. Transcripts for GFAP showed significant increase at all time points to varying levels while vimentin showed significant increase at all time points except for 2 hours in the cerebellum. GFAP mRNA levels did not show significant increase until 2 hours and increased moderately at 1 day, whereas it dramatically increased at 3 days and stayed high in the brainstem. However, vimentin levels showed immediate induction and progressive increase until reaching a peak at 7 days. In the midbrain, transcript levels for GFAP showed significant progressive increase until 1 day; the levels dropped slightly after that, but never returned to control levels at 7 days after treatment, whereas vimentin showed moderate increase throughout the time-course. In the spinal cord, GFAP mRNA levels showed progressive increase until 3 days and levels came down to control values at 7 days. Vimentin levels showed immediate induction, and levels stayed more or less the same, until slightly down-regulated but still over-expressed at 7 days.

induced levels of both GFAP and vimentin at 7 days. There are abundant citations in the literature that note early induction of GFAP at 1.5 hours (41), 2.0 hours (42), and 3 hours (43) resulting from different forms of CNS injury. In most of these studies the overexpression of GFAP increased over time and persisted for a while, very similar to current findings. Persistent neuropathological changes were noted as early as 1 day in the hippocampus, cortex, and thalamus in sarin-treated rats (44), and these pathological changes persisted until 7 days and beyond (90 days).

Vimentin has been found to be overexpressed more readily than GFAP after CNS injury (45) and also involved in proliferation (46) and glial scar tissue formation (47). However, it is also clear that both GFAP and vimentin are needed for the effective biphasic recovery involving alternating up- and down-regulation of these two key astrocyte genes, depending on tissue specificity (48). This continued expression of vimentin and GFAP in the tissues of sarin-treated rat CNS may indicate that the astrocytes in sarin-treated CNS were not going to degenerate but instead regenerate in such circumstances (49). It may also indicate the attempts of astrocytes to dedifferentiate to increase vimentin expression for survival (50). Hence, the protection of astrocytes in the CNS of sarin-treated rats may be operating via dedifferentiation (by overexpressing vimentin) and rapid recovery of GFAP-positive astrocytes, combined with the repopulating of GFAP-positive astrocytes (51). Thus, the continued over-expression astrocyte-specific GFAP and vimentin probably indicates the protective strategy of these tissues.

The cholinergic system plays a fundamental role in regulation of the ability to detect and process stimuli and in the allocation of resources for processing of competing stimuli in the nervous system (52). The early response after sarin treatment can be attributed to AChE inhibition, increasing acetylcholine levels (53), and corresponding induction of signal transduction processes (54). Several organophosphates, including sarin, can exert direct effects on multiple brain proteins besides their effects mediated through AChE inhibition (3,4). These include blockade of muscarinic receptors and activation or blockade of nicotinic, glutamatergic, dopaminergic, and gabaergic receptors (4,19,21,33,55–57). Astrocytes have receptors for and physiologically respond to ACh in culture (58). Thus, ACh could have a direct action on CNS astroglia via muscarinic receptor activation (4). It also may be responding to signals from highly active adjacent neurons and not from direct cholinergic stimulation.

Neuronal activation as noted during seizures increases extracellular levels of lactate, causing local decreases in pH, which could activate adjacent astroglia, leading to increased GFAP expression (59). Thus, the molecular cascades in specific regions of sarin-treated CNS may be different from each other (depending on the degree of responsiveness and other local factors determining the microenvironment), thus initiating distinct sequence of events that may very well lead to abnormal clinical manifestations.

It is noteworthy that CNS regions such as the cerebellum, which has sparse cholinergic innervation, also showed early induction and persistence of overexpression at 7 days for both GFAP and vimentin, thereby suggesting the important role of specialized cell types (Purkinje cells and Bergmann glia) and other non-cholinergic pathways in sarin-induced neurotoxicity. Studies on the effect of DFP on hen nervous system demonstrated that there was down-regulation in the transcript levels of GFAP and vimentin in susceptible regions; this was attributed to astrocyte loss and/or astrocyte dysfunction (19). Although we did not observe down-regulation of GFAP and vimentin transcript levels in more responsive tissues, such as brainstem and midbrain, we noticed absence of induction of GFAP and very moderate induction of vimentin at early time points in brainstem. In midbrain, induction of GFAP was very moderate at early time points, whereas induced vimentin levels remained moderately high throughout the time-course. Thus, both the brainstem and midbrain might have suffered astrocyte dysfunction and/or marginal astrocyte loss, as we have seen in susceptible tissues of DFP-treated hens (19). The differences noticed could be attributed to species-specific susceptibility and neurotoxicity, as well as drug specificity (2).

In summary, single dose of sarin ($0.5 \times LD_{50}$) showed mRNA induction of key astroglial marker genes, such as GFAP and vimentin, in the cortex, cerebellum, brainstem and midbrain, and spinal cord of the CNS. The induction of these genes showed distinct spatial/temporal differences for each tissue studied. Vimentin has been found to be more readily and immediately induced in most of the tissues than GFAP and persisted consistently until 7 days in most of the tissues. In contrast, GFAP was not induced immediately in brainstem, and the levels showed a downward trend at 7 days in cortex, spinal cord, and cerebellum. However, overall, it is also clear that both GFAP and vimentin are needed for the effective biphasic recovery involving alternating up- and down-regulation of these two key astrocyte genes, depending on tissue

specificity. The changes that we have seen in the transcript levels of GFAP and vimentin could be due to astrocyte dysfunction, astrocyte loss, accompanied by astroglial cell proliferation, dedifferentiation, and changes in functional state of neuronal cell types, thus altering the neuron-glia homeostasis.

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Review

Sarin: health effects, metabolism, and methods of analysis

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Abstract

Sarin (*O*-isopropylmethylphosphonofluoridate) is a highly toxic nerve agent produced for chemical warfare. Sarin is an extremely potent acetylcholinesterase (AChE) inhibitor with high specificity and affinity for the enzyme. Death by sarin is due to anoxia resulting from airway obstruction, weakness of the muscles of respiration, convulsions and respiratory failure. The main clinical symptoms of acute toxicity of sarin are seizures, tremors and hypothermia. Exposure to sarin during incidents in Japan in 1994, 1995 and 1998, and possible exposure to low levels of sarin during the Gulf War, resulted in the deaths and injury of many people in Japan and caused possible long-term health effects on Gulf War veterans. Symptoms related to sarin poisoning in Japan still exist 1–3 years after the incident and include fatigue, asthenia, shoulder stiffness and blurred vision. Sarin produced seizures in rats and pigs. Recent studies showed that long-term exposure to low levels of sarin caused neurophysiological and behavioral alterations. Toxicity from sarin significantly increased following concurrent exposure to other chemicals such as pyridostigmine bromide. Further research to examine effects of sarin on the cellular and the molecular levels, gene transcription, endocrine system as well as its long-term impact is needed. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Sarin; Nerve agents; Gulf War; Organophosphates

1. Introduction

Sarin is a highly toxic nerve agent produced for chemical warfare, first produced in Germany in 1937 (Somani, 1992; Bakshi et al., 2000a,b). Sarin produced seizure in rats and guinea pig (Shih and McDonough, 1999). The neurotoxicity of sarin is mediated by inhibition of acetylcholinesterase (AChE) activity (Prenant and Crouzel, 1990; Gupta et al., 1991; Khan et al., 2000, 2001; Young et al., 2001; Bakshi et al., 2000a; Jones et al., 2000). Immediate death from exposure to sarin occurs because of respiratory arrest (Rickett et al., 1986). Sarin can be inhaled or absorbed from the gastrointestinal tract and crosses the skin barrier and eyes (Fulco et al., 2000; Spruit et al., 2000). The adverse health and environmental impact of the use of sarin

could stem from its degradation products or from by-products generated through its synthesis (Munro et al., 1999; Li et al., 2000; Satoh and Hosokawa, 2000). Sarin was implicated in several incidents in Japan in 1994, 1995 and 1998 that resulted in the death and injury of many people. Also veterans may have been exposed to low levels of sarin during the Gulf War (Nakajima et al., 1998a; Noort et al., 1998; Abou-Donia, 1999; Fulco et al., 2000; Li et al., 2000; Suzuki et al., 2000; McCauley et al., 2001). Symptoms related to sarin poisoning in Japan still exist 1–3 years after the incident and include fatigue, asthenia, shoulder stiffness, blurred vision, and chronic decline in memory function (Nakajima et al., 1999; Nishiwaki et al., 2001). Troops in the Gulf War were treated with the soman prophylaxis, pyridostigmine, which is a carbamate anticholinesterase. As sarin exposure may have occurred, the effects of these two compounds administered together is clearly of interest, and in rats administration of a combined dose of 13 mg/kg of pyridostigmine bromide and 80 µg/kg of sarin produced severe tremors, seizures and convulsion (Abu-Qare and Abou-Donia, 2001a). Furthermore, exposure of rats through inhalation to low levels of 0.8–2.5 µg/l of sarin for up to 12 months produced behavioral, biochemical and neurophysiological effects (Kassa et al.,

Abbreviations: AChE, acetylcholinesterase; IMPA, isopropyl methylphosphonic acid; MPA, methylphosphonic acid; NTE, neuropathy target esterase; OPIDN, organophosphate induced delay neurotoxicity.

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Degradation products of sarin can cause toxic effects, as reported by Nijima et al. (1999), who showed that a sarin-like agent (bis(pinacolyl methyl)phosphonate) may stimulate a membrane tyrosine kinase, including growth factor receptors in rat brain.

3.2. Peripheral nervous system

The sequence of the effects of sarin on the peripheral nervous system depends on the route of exposure (Lotti, 2000). Examination of a 51-year-old man who inhaled sarin during an incident in Japan and died after 15 months, showed dying-back degeneration of the peripheral nervous system with symptoms such as marked nerve fiber decrease in the sural nerve, moderate fiber loss in the sciatic nerve, and unremarkable dorsal root ganglia, dorsal roots, and posterior column of the spinal cord (Himuro et al., 1998). Acute or subchronic doses of sarin significantly inhibited the m2 selective muscarinic acetylcholine receptor ligand binding in the cortex (Abou-Donia, 1999, 1994; Jones et al., 2000; Khan et al., 2000).

3.3. Organophosphate induced delay neurotoxicity (OPIDN)

OPIDN is a progressive neuropathy that manifests 1–4 weeks after an acute exposure to some organophosphates. It is a neurodegenerative disorder characterized by a delayed onset of prolonged ataxia and upper motor neuron spasticity from a single or repeated exposure to organophosphates. Symptoms include ataxia and flaccid paralysis (Abou-Donia and Lapadula, 1990). Symptoms may persist for 1 year or longer. Development of OPIDN depends on species, dose, route of exposure, and concurrent exposure to other chemicals (Abou-Donia et al., 1985). OPIDN is associated with axonal degeneration of the peripheral nerves and spinal cord (Abou-Donia and Lapadula, 1990; Abou-Donia, 1995; Weiner and Jortner, 1999). Neuropathy target esterase (NTE) has been proposed to be the initiation site of OPIDN. However, in some cases NTE activity in the brain returns to nearly normal levels before the onset of the neuropathy (Carrington and Abou-Donia, 1984). Furthermore, a study showed that exposure of the European ferret to triphenyl phosphine produced symptoms of OPIDN such as ataxia and paralysis without causing significant inhibition of NTE or AChE activities (Davis et al., 1999). Himuro et al. (1998) reported that a 51-year-old man who was exposed to sarin during the Tokyo subway incident died months later. Neuropathologic alterations were consistent with dying-back generation of the peripheral nervous system characteristics of OPIDN. In sensitive species, high doses of sarin can cause OPIDN, with ataxia and paralysis appearing days or weeks after exposure (Somani, 1992; Brown and Brix, 1998; Fulco et al., 2000). Chickens, the experimental animals for

OPIDN, given three doses of 250 µg/kg sarin over a 6-day period had brain NTE inhibited more than 90%, exceeding the threshold associated with the onset of OPIDN, but major signs of OPIDN did not even appear in hens after 3 weeks of dosing (Wilson et al., 1998). A recent study from our laboratory produced OPIDN in hens using a single intramuscular dose of 10 µg/kg sarin (0.5 LD₅₀) following exposure to pyridostigmine bromide, DEET and permethrin (unpublished data). However, several studies showed that sarin at doses between 61 and 400 µg/kg did not produce OPIDN in chickens (Gordon et al., 1983; Crowell et al., 1989). Fourteen days following administration of 5 mg/m³ of sarin by inhalation in mice daily for 10 days, Husain et al. (1993) reported symptoms of muscular weakness of the limb, slight ataxia, and significant inhibition of NTE in the brain.

The implications of exposure to sarin in OPIDN need further investigation using realistic doses and experimental models. Studies are needed to answer whether sublethal doses of sarin produced OPIDN when given with stress. Furthermore, whether concurrent exposure to sarin and other chemicals enhances its delivery to NTE, leading to its inhibition and aging, resulting in OPIDN.

3.4. Other effects

Inhalation exposure to sarin in male rats resulted in long-term depression of the immune system (Henderson, 2000; Kassa et al., 2000b). Abu-Qare and Abou-Donia (2001a) reported that concurrent exposure to 80 µg/kg of sarin and 13 mg/kg of pyridostigmine bromide increased levels of 8-hydroxy-2'-deoxyguanosine and 3-nitrotyrosine, biomarkers of oxidative stress in rat urine. Administration of 100–380 µg/kg of sarin orally in pregnant rats and rabbits caused significant maternal toxicity and increased maternal mortality, but there was no evidence of developmental toxicity (LaBorde et al., 1996). Kassa et al. (2000a) showed that low levels of sarin significantly decreased the incorporation of radiolabelled thymidine without changing total concentrations of DNA or proteins 3 months following exposure in rats. In vitro examination of victims after the Tokyo subway sarin poisoning showed elevated frequency of sister chromatid exchanges in lymphocytes (Li et al., 1998). Li et al. (2000) showed that by-products generated during sarin synthesis were cytotoxic to human lymphocyte cells in vitro. Subchronic doses of sarin produced effects on the blood-brain and blood-testes barriers in rats (Jones et al., 2000). Furthermore, Nieminen et al. (1990) found a decrease in locomotor activity up to 72 h after an intraperitoneal dose of 50 µg/kg of sarin in rats. Recent studies showed that the long-term (3, 6 and 12 months) exposure of rats to low levels of sarin caused behavioral and neurophysiological effects such as a decrease in activity and mobility, an altered gait, and an increase in the excitability of the nervous system (Kassa et al., 2001a,b).

factors such as heat, stress and diseases. The recent studies on the long-term impact of exposure to sarin (Kassa et al., 2001a,b) showed the need for more examination of this effect on cellular or molecular level and possible alteration of genes transcription. An additional area that needs to be explored is sarin's effect on the reproductive/developmental and endocrine systems. The incidents in Japan that involved sarin demonstrate that the danger of exposure to sarin is not only restricted to wars and conflicts, but could involve civilians in different parts of the world.

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Sarin (nerve agent GB)-induced differential expression of mRNA coding for acetylcholinesterase gene in the rat central nervous system.

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Running Title : AChE mRNA expression in sarin-treated rat nervous system

Abstract:

We carried out a time course study on the effects of a single intramuscular (i.m) dose ($0.5 \times LD_{50}$) of sarin (O-isopropyl methylphosphonofluoridate) which is also known as Nerve agent GB, on the mRNA expression of acetylcholinesterase (AChE) in the brain of male Sprague-Dawley rats. Sarin inactivates the enzyme acetylcholinesterase (AChE) which is responsible for the breakdown of the neurotransmitter acetylcholine (ACh), leading to its accumulation at ACh receptors and overstimulation of cholinergic system. Rats were treated with $50 \mu\text{g/kg/ml}$ of sarin ($0.5 \times LD_{50}$) and terminated at the time points 1, 2 hr and 1, 3, and 7 days post-treatment. Control rats were treated with normal saline. Total RNA was extracted and northern blots were hybridized with cDNA probes for AChE and 28S RNA (control). Poly-A RNA from both treated and control cortex were used for RT-PCR based verification of northern data. The results obtained indicate that a single (i.m) dose of sarin ($0.5 \times LD_{50}$) produced differential induction and persistence of AChE mRNA levels in different regions of the brain. Immediate induction of AChE transcripts was noted in brainstem ($126 \pm 6 \%$), cortex ($149 \pm 4 \%$), midbrain ($153 \pm 5 \%$) and cerebellum ($234 \pm 2 \%$) at 1 hr. AChE expression level, however, increased overtime and remained elevated after a decline at 1 day in the previously shown more susceptible brainstem. The transcript levels remained elevated at later time point (3 days) in the midbrain, after a dramatic decline at day 1 ($110 \pm 2 \%$). In the cortex, transcript levels came down to control values by day 1. The cerebellum also showed a decline of the elevated levels observed at 2 hrs ($275 \pm 17 \%$) to control values by day 1. RT-PCR analysis of the AChE transcript at 30 minutes in the cortex showed an induction of $213 \pm 3 \%$, of control levels, confirming the expression pattern obtained by northern blot data. The immediate induction followed by the complex pattern of AChE mRNA time course in the CNS may indicate the activation of both cholinergic-related and

unrelated functions of the gene playing an important role in the pathological manifestations of sarin-induced neurotoxicity.

Key words: sarin, acetylcholinesterase, mRNA expression, central nervous system, acute exposure.

Abbreviations:

AChE, Acetylcholinesterase; CNS, Central Nervous System; ACh, Acetylcholine; ChE, Cholinesterase; APS, Ammonium persulphate; BuChE, Butyrylcholinesterase; nAChR, Nicotinic acetylcholine receptor; CRE, cAMP-responsive element. mAChR; Muscarinic acetylcholine receptor; DECP, Diethylpyrocarbonate; DFP, Diisopropylphosphorofluoridate; IEG, Immediate Early Genes; GFAP, Glial Fibrillary Acedic Protein; ChAT, Choline acetyl transferase; VACHT, Vesicle acetylcholine transporter; GAPDH, Glyceraldehyde-3-Phosphate dehydrogenase; p-CREB, Phosphorylated cyclic AMP-response element binding protein; PKA, Protein Kinase A; TEMED, Tetramethylethylenediamine; TBE, Tris Borate EDTA buffer.

1. Introduction:

Sarin (*O*-isopropyl methylphosphonofluoridate) is an organophosphorus ester that was developed as a warfare agent and also known as nerve agent GB [1]. Sarin is suspected to be one of the several chemicals to which several thousands Gulf-War veterans were possibly exposed [2]. We have shown that sarin-induced neurotoxicity involves multiple mechanisms [3-5]. Cholinergic inhibition plays an immediate, central and persistent role in the development of sarin-induced neurotoxicity, even though there may be convergence and / or cross talk between several signaling pathways [3], [5], [6]

Hydrolysis of the neurotransmitter acetylcholine (ACh) is the principal step that terminates the intercellular communication pathway defined as cholinergic signaling. This pathway includes neuromuscular and inter neuronal transmission, operates through a variety of receptor subtypes, and activates different intracellular responses in multiple tissues and cell types. Acetylcholinesterase (AChE) is the enzyme that performs this essential step in humans and in other species.

AChE degrades ACh neurotransmitter rapidly [7,8]. This enzyme is also present in non-neuronal cells [9] and in neurons assumed to be non-cholinergic, such as dopaminergic neurons in the substantia nigra [10]. The wide range of cellular distribution of AChE, from meninges to various apparently non-cholinergic glia and neuronal cells, suggests additional important roles for this enzyme in the nervous system. For example, cell types that are known to receive cholinergic input yet contain no AChE include spiny neurons in the striatum [10]. In contrast, cholinceptive cells that can be excited by ionophoretic ACh (for example, Purkinje cells in the cerebellum) exist, but appear to lack AChE and do not receive cholinergic input in their mature state [11]. Furthermore, identification of newer roles for cholinergic signaling such as hormonal

control of hydration levels [12] add complexity to AChE function. Statistically significant correlation between body weight loss and plasma cholinesterase levels of the sarin dosed animals has been shown recently, thus establishing the link between sarin, AChE and weight loss [13]. This above mentioned study also confirmed the established fact that satiety center controlling the hypothalamus and CNS may thus be affected by sarin treatment via cholinergic pathways. Similarly newer functions of AChE such as neurite outgrowth [14], neuronal differentiation [15] make it as a key gene whose expression may have diverse and important role in the recovery or degeneration of different cell types in the CNS after neurotoxic insult. Thus, studying the mRNA expression pattern of AChE gene in sarin treated brain of rats will give more insight into its presumed central role.

2. Materials

Sarin (GB) was obtained from U.S.Army Medical Research and Material Command, Fort Detrich, Frederick, MD. Radioactive [$\alpha^{32}\text{-P}$] dATP (3000ci/mmol) was purchased from New England Nuclear (Boston, MA). Duralon-UV membranes were purchased from Stratagen (La Jolla, CA) and Random Primer labeling system was obtained from Invitrogen: Life Technologies; Carlsbad, CA. Other chemicals used were purchased from standard sources.

3. Methods

3.1 Animal Treatment:

Young adult male Sprague-Dawley rats, weighing approximately 250 grams were purchased from Charles River, Raleigh, NC . Rats were kept in a temperature-controlled room at 21-23 °C with a 12 -hour light-dark cycle. They were provided with Rat Chow (Purina, St.Louis, MO) and tap water Libitum. Animal treatment with sarin was carried out in specially designated treatment room at Duke University. All animal treatments and procedures were approved by the Duke University Institutional Animal Care and Use committee and to the recommended guidelines by the U.S. Army. We used the minimum number of animals necessary to produce reliable scientific data. Groups of five rats were treated with a single intramuscular injection of 50 $\mu\text{g/kg}$ in normal saline 1ml/kg into the thigh muscle, for each time point. The control group containing five rats was injected with 1ml/kg saline as described above. The rats were examined daily for any clinical signs and weighed two times a week. At the termination of the experiment, the animals were anesthetized with 100 $\mu\text{g/kg}$ of ketamine/xylazine and the animals were dissected; the brain was removed and washed thoroughly with ice-cold diethylpyrocarbonate (DEPC)-treated water to remove traces of blood. The rats were sacrificed at 1, 2 hrs as well as 1, 3 and 7 days post sarin-treatment. The different brain regions, i.e.; cortex,

cerebellum, brainstem and midbrain were quickly dissected at the end of each time point and frozen in liquid nitrogen. The tissues were kept at -70°C until used for total RNA extraction.

3.2 Preparation of cDNA probes:

Rat AChE cDNA was a gift from Dr.C.Legay of Laboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS URA 1875, 75230 Paris, Cedex 05, France. 28S RNA cDNA probe was purchased from ATCC (American Type Culture Collection, Rockville, MD). The clones were amplified and cDNA was purified according to Ausubel et al (1995) [16]. The cDNA insert for AChE hybridization was prepared by Hind III and Xba digestion, while Eco RI digestion was used for 28S RNA insert preparation from its vector. The inserts were gel purified using standard protocols [16].

3.3 Total and Poly-A RNA extraction:

Total RNA was purified from cortex, cerebellum, brainstem and midbrain from control and sarin-treated rats by Trizol kit from (Invitrogen: Life Technologies; Carlsbad, CA). Poly-A RNA was prepared from the cortex of both control and treated rats, following the modified method of Chomczynski and Sacchi [17]. Briefly, a denaturing solution (250g guanidine thiocyanate dissolved in 293 ml of double distilled water and 17.6 ml of sodium chloride, pH, 7, along with 26.4 ml of 10% sarcosyl, together heated up to 65 °C and added with 360 µl of β-mercaptoethanol) was used to homogenize the tissue and then extracted with phenol and chloroform and purified using oligo-dt columns. The purity and quantity of the RNA was determined by A_{260}/A_{280} ratios and then A_{260} respectively using UV-1601 Shimadzu Spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

3.4 Reverse transcription:

Using a first strand cDNA synthesis kit (Invitrogen, Life Technologies; Carlsbad, CA), 1-2 µg of RNA was reverse transcribed by AMV reverse transcriptase. Reverse transcription was primed with random hexamers or oligo-(dt) in a volume of 15 or 33 µl. Polymerase chain reactions were performed with 2-4 µl of the resulting cDNA solution.

3.5 Polymerase Chain Reaction (PCR):

Oligonucleotide primers for the PCR experiments were made in an automated DNA synthesizer in the DNA core facility at Duke University. With the aid of Seqweb primer designer program of Duke University Shared Bioinformatics Resource, primers for AChE and cyclophilin were designed. The forward primers used for AChE amplification was 5' GAC TGC CTT TAT CTT AAT GTG 3' and the reverse primer was 5' CGG CTG ATG AGA GAT TCA TTG 3'. The primers for the hybrid product consisting of cyclophilin with the flanking AChE sequences at both the ends were as follows: Forward primer was 5' TCT CCT TCT TTG CTC AGC GAC TTA AAC CCC ACC GTG TTC TTC 3' and the reverse primer was 5' GTT CCC GTC ACA GGT CTG AGC AAT GCC CGC AAG TCA AAG A 3'. DNA was amplified in a Perkin Elmer-Cetus thermocycler 9700 version. Reaction tubes contained, in a final volume of 50 µl, 5 µl of cDNA, 0.5 U of AmpliTaq DNA polymerase, 200 µM dNTPs and 8 pmole of primers in PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3). Radioactive dCTP was used for PCR reactions. Each PCR cycle consisted of denaturation temperature: 94 °C, annealing temperature: 55°C, extension temperature: 72 °C. The PCR products were separated using 16 µl of DNA on 5% polyacrylamide gel consisting of polyacrylamide, TEMED, TBE, and APS, electroblotted overnight and exposed to X-ray films. For determining the ideal cycle number for the quantitative analysis, the PCR samples were taken out at different cycle numbers such as 18, 21,

24, 30, 35, 40 and run on 1% agarose gels and quantified and plotted to decide the linearity of the amplification. PCR reactions have given maximum yield in a linear range at 30 cycles and hence all other experiments were done for 30 cycles. A total number of 5 animals were used for controls as well as sarin-treatment.

3.6 Data analysis:

Each autoradiogram of the gel was scanned into Photoshop 4.0 using a UMAX Astra 2400S Scanner. The bands were quantified using IP-Lab gel of molecular dynamics and data the results were presented as percentage values of the controls. The value obtained for AChE was divided by that of cyclophilin (internal control). Statistical significance was calculated using student's t test. P value < 0.05 was considered significant. The radioactive bands were left in the bioimaging system until saturation point.

3.7 DNA Sequencing:

Amplified DNA from the PCR reaction were cloned using One-shot PCR cloning kit (Invitrogen: Life Technologies; Carlsbad, CA) and the cloned products were sequenced using Duke University Core Sequencing Facility. Sequence identity was verified using several software programs provided by Duke University Shared Bioinformatics Program.

3.8 Northern Blot Hybridization

Twenty micrograms of total RNA from each tissue was used for agarose gel electrophoresis and transferred to Duralon nylon membranes. The membranes were hybridized with ³²P labeled cDNA probes and latter were labeled by Random Primer labeling system using [α -³²P] dATP as the radioactive nucleotide. The blots were exposed to phosphorimaging plates and radioactivity in the bands was quantified with Imagequant System (Molecular Dynamics, Inc; Sunnyvale, CA). Radioactivity (PSL values) of mRNA bands from each tissue for each time

point (1 hr to 7 days) was calculated as the percentage of value obtained for the same tissue from control rats. The radioactivity of the mRNA bands from control tissues for any probe and tissue was assigned as 100% . The radioactive bands were left in the bioimaging system until the saturation point.

3.9 Statistical analysis:

The hybridized blot was exposed to phosphorimaging plates at least two times and the radioactivity of band was quantified and normalized as described alone. The results were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test. P value < 0.05 was considered significant.

4.Results:

4.1 Clinical Signs:

All animals were observed for the development of clinical signs of sarin toxicity. All the animals treated with 0.5 X LD₅₀ were inactive until four hours after treatment. There were no observable changes in clinical condition thereafter.

4.2 mRNA expression of AChE in the cortex of sarin treated rats:

In the cortex, a single i.m. dose of sarin (50 µg/kg) produced in the cortex a significant increase in AChE transcript level to 149±4 % at 1 hr and remained more or less at the same level (147±3%) at 2 hrs. This level, however was down-regulated to 110±2 % after 1 day which stayed at the same level for 3 days (110±3%) and 7 days (100±2%). The expression of 28S RNA was used as a control to reveal any variation in AChE expression due to purity of RNA or error in estimation and loading. The same northern blot was hybridized to both of the probes and the blot was stripped after hybridization with each probe. The 28S RNA did not show any significant change in their expression at any time point after sarin treatment. Alteration in the expression of AChE was real and could not be ascribed to the above mentioned errors, since the time-course profile was different for both the genes. The mRNA expression data of AChE were normalized to control 28S RNA in all the tissues (Fig 1; Fig 2A).

4.3 RT-PCR of the AChE transcript in the cortex:

A single dose of sarin (0.5 X LD₅₀) at 30 minutes post-treatment resulted in a statistically significant induction of AChE transcripts in the cortex to 213±3 % of the control level (Fig 3A and 3B).

4.4 mRNA expression of AChE in the cerebellum of sarin treated rats:

The AChE transcript levels showed early dramatic induction in the cerebellum at 1 hr

($234\pm2\%$) that remained elevated until 2 hr ($275\pm2\%$). These elevated levels were the highest at all of the time points of all the tissues studied. The transcript levels for AChE declined and was the same at 1 ($104\pm3\%$), 3 ($112\pm4\%$) and 7 day ($109\pm6\%$) post-treatment. There was no alteration in 28S RNA levels at any time point in sarin treated cerebellum (Fig 1; Fig 2B).

4.5 mRNA expression of AChE in the brainstem of sarin treated rats:

The immediate induction levels of AChE in the brainstem ($126\pm6\%$) were further increased to $157\pm8\%$ at 2 hrs. Although there was a decline to $139\pm7\%$ at day 1, the transcript levels remained more or less the same at 3 ($137\pm3\%$) and 7 days ($132\pm7\%$). There was no alteration in 28S RNA levels at any time point in sarin-treated brainstem (Fig 1; Fig 2C).

4.6 mRNA expression of AChE in the midbrain of sarin treated rats:

There was a significant increase in AChE transcript levels ($153\pm5\%$) at 1 hr which remained high at 2 hr ($160\pm5\%$) in the midbrain. The transcript levels eventually came down to near control values at 1 day ($110\pm2\%$). There was a statistically significant increase, at 3 days ($118\pm3\%$) and the level was back to near control values ($113\pm5\%$) at 7 days. There was no alteration in 28S RNA levels at any time point in sarin-treated rat CNS as compared to the controls (Fig 1; Fig 2D).

5. Discussion:

AChE gene expression is an important part of cholinergic neuronal systems for the maintenance of CNS homeostasis. Many components of cholinergic neural systems have not been investigated at the level of the gene, and the nature of regulatory mechanisms which foster coordinated expression of cholinergic macromolecules remain less explored. Hence our current study provides the preliminary evidence that AChE gene expression is differentially modulated in different regions of the brain after sarin treatment, thus confirming the complexity of cholinergic regulation.

We have shown earlier that diisopropyl phosphorofluoridate (DFP) a structural analog of sarin, induced some of the immediate early genes (IEG) such as c-fos [18] and c-jun [19] as well as glial fibrillary acidic protein (GFAP) and vimentin[20]. Our recent studies on the effect of sarin on the mRNA expression of astroglial markers such as Glial Fibrillary Acetic Protein (GFAP) and vimentin suggest that the immediate induction of these genes could be due to the presence of binding sites for IEGs such as c-fos and c-jun. Similarly the presence of c-Fos binding sites in the promoters of key cholinergic genes, such as the genes encoding AChE , ChAT and the vesicular acetylcholine transporter (VACHT), indicate that elevated c-Fos levels might activate regulatory pathways leading to long-term changes in the expression of proteins mediating brain cholinergic transmission [21]. These acute cholinergic stimulations promote selective bi-directional changes in the expression of the genes regulating acetylcholine metabolism. Exposure of adult mice to the organophospharous insecticide fenthion resulted in AChE overproduction [22], which has been reported to cause retinal degeneration [23]

It is interesting to note that in the present study, the previously shown, more susceptible tissue, brainstem [3] exhibited low levels of immediate induction of AChE gene expression, when

compared to other CNS regions, which was followed by persistent levels latter on. The inhibition of AChE activity was shown to be the maximum at 3 hrs in the brainstem at 0.5 X LD₅₀, reaching control levels at 24 hrs. AChE gene expression can be correlated to the highest inhibition of AChE and ChAT at early time points, its recovery to control levels at 24 hrs [3] as well as to the increased enzyme activity at similar dose at later time point (15 days) [24]. It is also interesting to note that similar dose of sarin also caused a differential induction of the astroglial marker genes such as GFAP and vimentin [6] and neuronal marker, alpha tubulin[5]. In all of these scenarios, it was brainstem that showed absence of induction at early time points, followed late induction. Taken together, there is a direct inhibitory effect of sarin on the brainstem, which not only severely inhibits the cholinergic pathways, but also activates proteases that may lead to synapse loss [25] in an activity-dependent manner. This could result in significant amount of cell death or cell injury. In a similar way, immediate induction and persistence of induced levels of AChE mRNA in midbrain can be correlated with initial inhibition of AChE enzyme levels [3], followed by the increase of enzyme activity at 15 days [24]. This modified levels of transcripts can also be explained by the combined effect of direct inhibition of cholinergic pathway coupled with the activation of the other apoptotic or necrotic pathways.

In spite of the high amount of cholinergic pockets, cortex showed only a moderate level of induction at 1 hr that persisted until 2 hrs and eventually came down to control levels at 1 day. On the other hand, a higher level of induction (212%) at 30 minutes, indicates the efficiency of the feedback mechanisms in bringing down the levels latter on. The AChE enzyme activity remained inhibited at early time points [3] as well as later time points (until 15 days) [24]. As mentioned earlier this efficient feedback system at transcriptional level in the cortex as suggested

by Kaufer et al, [21] may be modulating the AChE mRNA expression levels.

The most interesting aspect of the AChE expression in sarin-induced changes in the brain is that the cerebellum showed the maximum induction of AChE immediately and its levels persisted until 2 hrs before coming to basal levels. It is possible that AChE overexpression in cerebellum may be the classic scenario, where AChE may be switching into non-catalytic functions such as neurite extension [26], interneuronal interactions [27] and neuronal apoptosis [28]. There have been reports of transient expression of AChE in developing embryos [22]. Recently, we have shown that rats treated with 0.5 X LD₅₀ sarin exhibited decreased plasma BuChE, an increased Blood brain barrier (BBB) permeability in the midbrain and the brainstem, and degeneration of Purkinje neurons in the cerebellum [29]. We have shown earlier that cerebellum showed immediate induction of IEGs more readily than other CNS regions in DFP-treated hens [19]. We have also shown that DFP differentially modulated levels of PKA [30] and pCREB [31] in the CNS of hen. Choi et al [32] showed that PKA mediates the expression of AChE via CRE (cAMP-responsive element). Taken together, it is conceivable that the PKA / pCREB / IEGs / pathway acts on several proteins including AChE which may be playing the key role in the pathophysiology of long-term clinical development. Downstream pathways may be of both degenerative and regenerative types as mentioned above.

At this point it is difficult to speculate whether the stability of the AChE transcript or the rate of the transcription plays an important role in the differential induction of AChE mRNA, although the stability of the transcript have been reported to be playing crucial role [33,8]. In addition, neurons express two pools of AChE : active and inactive [34]. How these enzyme pools are regulated during the post-translation process is not known. Moreover, Kaufer et al [35] suggested that in addition to the effects of ACh, an autologous feedback response could regulate

transcriptional elevation from the AChE gene through AChE-anti-ChE complexes acting on signaling intracellular pathways. Complexes of an extracellular protein like AChE could affect intracellular transcriptional signals through their similarity to complexes of neuronal proteins with extracellular domains resembling AChE in their sequence and presumed folding properties [36]. It is also shown that in addition to its catalytic capacity, AChE interacts with target proteins on the neuronal plasma membrane, which can transduce intracellular signals. Such interactions might explain the intensity of the anti-ChE responses, as they add upon the signals induced by the elevated ACh levels.

In summary, a single i.m. dose of 0.5 X LD₅₀ sarin treatment resulted in differential induction of AChE mRNA in different regions of the brain. The immediate induction followed by the complex pattern of time course in the AChE mRNA levels of the brain indicates the activation of both cholinergic-related and unrelated functions of the gene plays an important role in the long-term pathological manifestations of sarin-induced neurotoxicity. Sarin-induced AChE gene expression may explain, at the best in part, our recent findings that acute sarin exposure caused neuronal death of specific regions in the rat brain [37].

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Figure Legends:

Figure 1:

Northern blots showing the expression of AChE and 28S RNA in the central nervous system of sarin-treated rats. Rats were treated with a single dose of sarin (0.5X LD₅₀) and sacrificed at 1 and 2 hrs as well as 1, 3, and 7 days. Total RNA (20µg) from cortex, cerebellum, brainstem and midbrain were used as described in materials and methods. The ³²P-labeled probes prepared from cDNA inserts of rat AChE and 28S RNA were used. Two major bands of 3.8 Kb and 2.4 kb sizes were used for AChE transcript quantitation. Hybridizing bands were quantified using Imagequant system (Molecular Dynamics, Inc; Sunnyvale, CA). Radioactivity (PSL values) of mRNA bands were calculated as the percentage of value for the same tissue from the control rats. The radioactive bands were left in the bio-imaging system until they reached saturation point. The blots were stripped off of one probe and used for the next one. This figure shows only the relevant portion of representative autoradiograms, although five rats were used in each treatment group. There was a distinct differential expression pattern of transcripts in different regions of the CNS.

Figure 2: Expression of AChE and 28S RNA in different regions of the CNS:

Rats were treated with a single dose of sarin (50 µg / kg / ml) and sacrificed after 1, 2 hr and 1, 3, 7 days post-treatment. Total RNA was purified from cortex, cerebellum, brainstem and midbrain and 20 µg was used for the northern blotting using standard techniques and hybridization was carried out as described in materials and methods. AChE and 28S RNA cDNA probes represent the means±SEM of the percent of control values from untreated rats. The radioactivity of bands was measured in PSL units by exposing northern blots to phosphor-image plates, followed by quantification with the Fujix Bio-imaging system. Data from control animals

were taken as 100%.. Five rats were used in each group, while control group consists of 5 rats. Significant difference ($P<0.05$) from the control is marked with an asterisk. There was a statistically significant increase in AChE mRNA levels at 1 hr for all of the tissues, although the induced levels were small in the brainstem and the highest in the cerebellum. Both the cortex and the cerebellum continued to show induced levels at 2 hrs before reaching control values by 1 day and stayed there for 3 and 7 days. In contrast, brainstem and midbrain persistent induced levels at later time points to varying degrees.

Figure 3: RT-PCR analysis of the AChE transcripts in the cortex of sarin-treated rats:

A) Poly-A RNA was prepared from the cortex of the control and sarin-treated rats and cDNA synthesis was done. RT-PCR reactions were done for 30 cycles using the conditions mentioned in the materials and methods. The products were run in an 5% acrylamide gel and electroblotted overnight, and exposed to X-ray films and the bands were scanned into photoshop version 4.0 . The bands were quantified using IP-Lab Gel of Molecular Dynamics. The experiments were done in a group of five control and five treated animals. The data is presented as a percentage of controls. The values were means of five animals with standard error. Statistical analysis using an unpaired student's t-test has shown a significant increase ($213\pm30\%$) in transcript levels at 30 days in the cortex of sarin-treated rats.

B) Gel pictures showing the top hybrid band (consisting of cyclophilin flanked by AChE sequence) and the lower AChE specific band in the control and treated animals. The radioactive bands were left in the bio-imaging system until they reached saturation point.

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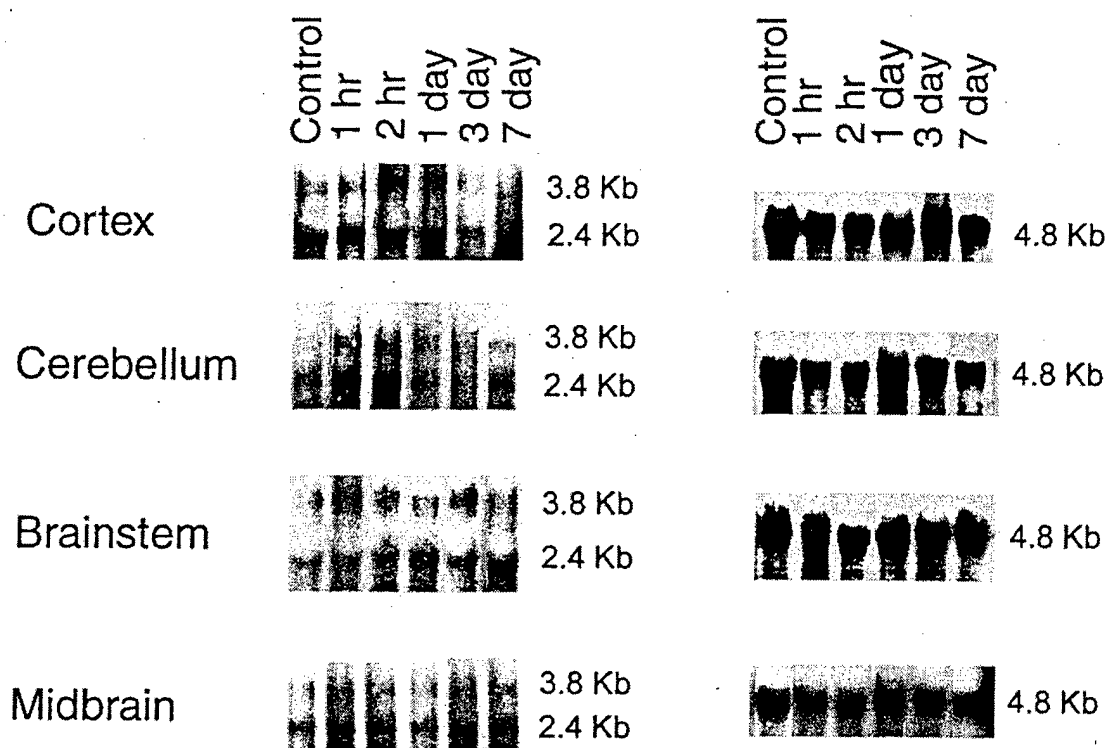
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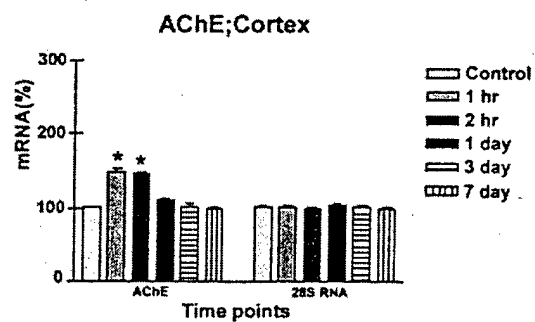
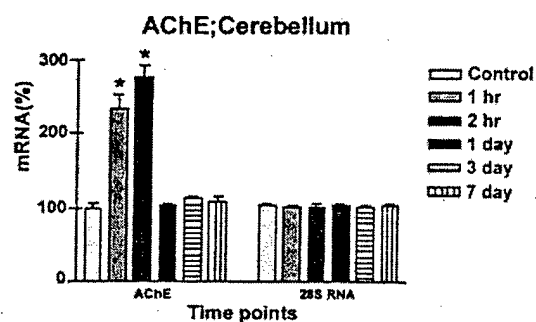
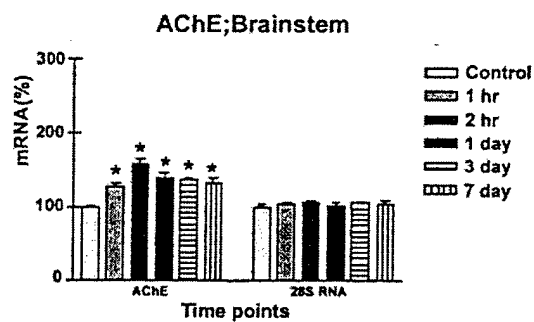
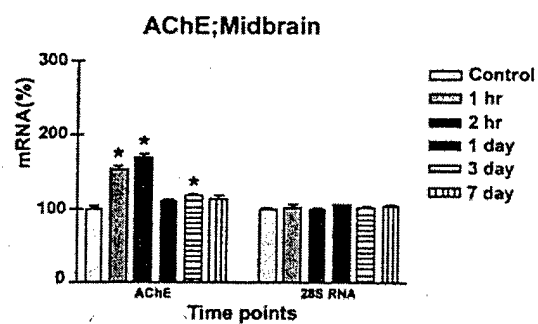
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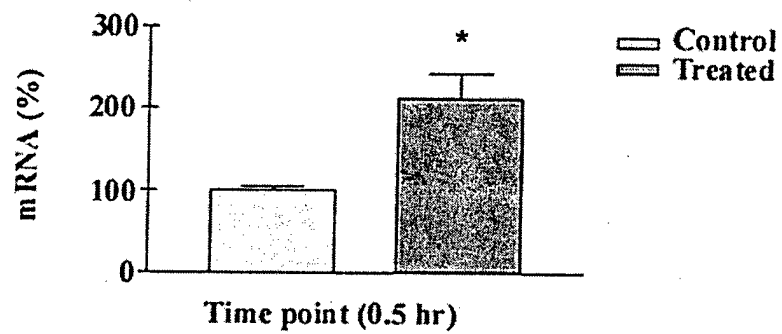
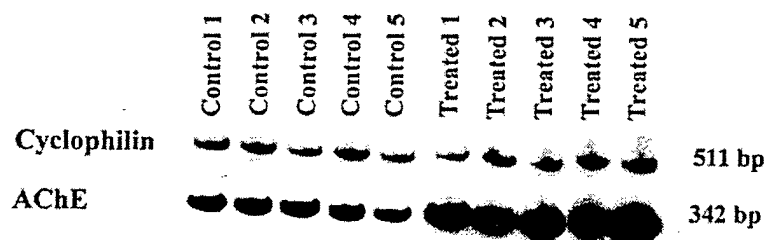
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AChE

28S RNA



A**B****C****D**

A**AChE; Cortex****B**

Abbreviations:

AChE, Acetylcholinesterase; CNS, Central Nervous System; ACh, Acetylcholine; ChE, Cholinesterase; APS, Ammonium persulphate; BuChE, Butyrylcholinesterase; nAChR, Nicotinic acetylcholine receptor; CRE, cAMP-responsive element. mAChR; Muscarinic acetylcholine receptor; DECP, Diethylpyrocarbonate; DFP, Diisopropylphosphorofluoridate; IEG, Immediate Early Genes; GFAP, Glial Fibrillary Acedic Protein; ChAT, Choline acetyl transferase; VACHT, Vesicle acetylcholine transporter; GAPDH, Glyceraldehyde-3-Phosphate dehydrogenase; p-CREB, Phosphorylated cyclic AMP-response element binding protein; PKA, Protein Kinase A; TEMED, Tetramethylethylenediamine; TBE, Tris Borate EDTA buffer.